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Nonviral Vectors for Gene Therapy: Physical Methods and Medical Translation

edited by
Leaf Huang
Dexi Liu
Ernst Wagner

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Nonviral Vectors for Gene Therapy

Physical Methods and Medical Translation

Edited by

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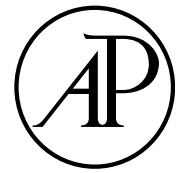

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DEDICATION

We dedicate this book to Professor Feng Liu, who was murdered on July 24, 2014, for his contribution in establishing the procedure of hydrodynamic gene delivery, the most effective and simplest nonviral method of hepatic gene transfer *in vivo* developed so far.

Huang, Leaf
Liu, Dexi
Wagner, Ernst

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CONTENTS

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CHAPTER ONE

Physical Methods for Gene Transfer

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Contents

Abstract

The key impediment to the successful application of gene therapy in clinics is not the paucity of therapeutic genes. It is rather the lack of nontoxic and efficient strategies to transfer therapeutic genes into target cells. Over the past few decades, considerable progress has been made in gene transfer technologies, and thus far, three different delivery systems have been developed with merits and demerits characterizing each system. Viral and chemical methods of gene transfer utilize specialized carrier to overcome membrane barrier and facilitate gene transfer into cells. Physical methods, on the other hand, utilize various forms of mechanical forces to enforce gene entry into cells. Starting in 1980s, physical methods have been introduced as alternatives to viral and chemical methods to overcome various extra- and intracellular barriers that limit the amount of DNA reaching the intended cells. Accumulating evidence suggests that it is quite feasible to directly translocate genes into cytoplasm or even nuclei of target cells by means of mechanical force, bypassing endocytosis, a common pathway for viral and nonviral vectors. Indeed, several methods have been developed, and the majority of them share the same underlying mechanism of gene transfer, i.e., physically created

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transient pores in cell membrane through which genes get into cells. Here, we provide an overview of the current status and future research directions in the field of physical methods of gene transfer.

1. INTRODUCTION

The concept of gene therapy has been first introduced in 1960s (Lederberg, 1963), and since then, the field has grown immensely despite the disappointing outcomes of the early clinical trials. The original concept of gene therapy has been redefined by the ever-expanding research in the field to include recently emerged therapeutic "molecular" strategies that center on the use of various forms of nucleic acids as agents for disease treatment, such as RNA interference (siRNA) and antisense oligonucleotides. At first, gene therapy aimed to treat diseases through intracellular gene delivery to restore missing gene function in patient's cells. However, the rationale, has recently evolved beyond the treatment of diseases to include prophylactic strategies, such as DNA vaccine, as well as diagnoses and gene marking (Barese & Dunbar, 2011). Clearly, the biomedical applications of gene therapy mentioned thus far encompass "gene delivery" as a cornerstone to accomplish the designated goal. Therefore, efficient gene delivery is essential in successful implication of gene therapy for human disease management. Gene delivery refers to the strategy in which genes or oligonucleotides are purposely introduced into cells in culture, animals, or humans to express the encoded information.

Current progress in gene therapy is challenged by the limited efficiency of gene delivery as a result of a series of intracellular and extracellular barriers limiting the amount of DNA that reaches the nuclei of target cells where gene expression takes place. This is because nucleic acids are large anionic molecules, rendering them hard to permeate through cell membranes. Moreover, biodegradation of naked DNA by serum and tissue nucleases poses another challenge to overcome (Wiethoff & Middaugh, 2003). Therefore, the development of delivery systems that are able to effectively and safely introduce DNA into host cells *in vitro*, and importantly, *in vivo* is critically needed. Immense research has been conducted to optimize gene transfer with acceptable safety and efficiency, and different strategies have been developed utilizing biological, chemical, or physical principles. Table 1 summarizes the characteristics of the methods employed thus far including viral and synthetic vectors and physical methods.

Table 1 Characteristic features of major classes of gene delivery systems

Method	Principle	Advantages	Disadvantages
Viral methods	Transfer of DNA or RNA through the natural viral infectious pathway using replication-incompetent viruses.	• Relatively high transduction efficiency and persistent gene expression. • Can be used with dividing and nondividing cells. • Highly effective in *in vivo* and *in vitro* trials.	• Strong induction of immune response. • Oncogenesis and insertional mutagenesis. • High cost. • Restrictions on the size of transgene.
Chemical methods	Transfer of DNA or RNA in complex with cationic lipids or polymers through cellular endocytosis pathway.	• Much safer and cheaper than viral vectors. • Amenable for chemical modification for targeted delivery. • Common and effective in *in vitro* experiments.	• Short duration of gene expression. • Low transfection efficiency in *in vivo* systems. • Low efficiency in nondividing cells.
Physical methods	Transfer of DNA or RNA through transient pores in plasma membrane created by mechanical forces.	• Can be used effectively in *in vitro* and *in vivo* experiments. • Specific tissue transfection. • Can be used with dividing and nondividing cells.	• Local tissue damage at the site of application. • Specialized instrument may be required. • Optimized procedure parameters are required for different types of tissues.

Viral vectors harness the natural infectivity of viruses to deliver genetic material to cells. Retroviruses and adenoviruses represent the most employed viral vectors in clinical trials (Edelstein, Abedi, & Wixon, 2007). The major limitations of viral vectors are their intrinsic properties of immunogenicity and potential harm of random insertion, such as activation of naturally silenced genes like oncogenes (Kay et al., 2001). Nonviral methods, on the other hand, utilize natural or synthetic compounds to

deliver genetic material into target cells. Chemical methods aim to formulate DNA in complexes to protect DNA from nuclease degradation and facilitate gene transfer by triggering internalization function of cells such as endocytosis, phagocytosis, and pinocytosis. Chemical systems are generally less immunogenic and safer than viral vectors and are increasingly employed in gene therapy studies, despite lower transfection efficiency (Al-Dosari & Gao, 2009; Gao et al., 2007; Mintzer & Simanek, 2009). Chemical systems are also amenable for modifications to enhance targeting specificity. Yet, further optimization is needed to enhance the delivery efficiency.

Apart from vector-based systems, physical methods of gene delivery are unique in eliminating the need for a special carrier to transfer DNA into cells. The physical methods employ physical forces to create transient pores in plasma membrane so that "naked" DNA molecules can pass through. Among the forces that have been utilized are electrical pulses, ultrasound waves, hydrodynamic pressure, and others (Villemejane & Mir, 2009).

Physical methods of gene transfer become increasingly applied in biomedical research due to its safety and simplicity in comparison to the other methods, and importantly, its ability to manipulate procedure parameters toward specific therapeutic needs. However, gene transfer efficiency of most physical methods is inferior to that of viral methods. Moreover, it becomes challenging when the internal organs are the primary tissues to be targeted for gene delivery, because it usually requires an invasive procedure to access target tissue (Kamimura & Liu, 2008). This chapter aims to provide an overview of the implied principles and techniques, the current status of various physical methods of gene transfer that have been developed, and the advantages and limitations of each method. In addition, we provide our perspective on future directions and how to address remaining challenges that restrict their applications in biomedical research and clinical practice.

2. MOST COMMONLY USED PHYSICAL METHODS

The barrier function of cell membrane is attributed to the dynamic nature of the membrane bilayer held together primarily by hydrophobic interaction of phospholipids, membrane proteins, and cholesterol. The rationale of physical methods for gene delivery at cellular level is to overcome membrane barrier and facilitate gene transfer into cells by generating transient pores or defects in plasma membrane through which DNA can get into cells. Importantly, these pores are transient in nature because, when membrane is broken open, the hydrophobic boundaries created are not

stable in aqueous environment and reseal quickly to limiting leakage of cellular content, while allowing DNA diffuse into cells. The following are a brief description of each of the physical methods developed and Figure 1 presents the milestones that contributed to the successful implementation of physical methods of gene transfer in gene therapy trials.

2.1 Needle Injection

Practically, the simplest intracellular gene delivery would be a direct injection of DNA into cells. Indeed, direct injection of DNA into the cell cytoplasm or nucleus by means of microneedle has been a common practice since 1980s (Capecchi, 1980). However, minimal progress has been achieved toward *in vivo* application. This is largely due to the technical difficulties in the procedure, making it extensively laborious because only one single cell can be injected at a time. Moreover, specialized tools are needed to perform injection, such as glass microneedle, micropipette, and precise-positioning manipulator to control the movement of the micropipette. Typically, all the work is carried out under a proper microscope. Though, the high efficiency of gene transfer triggers outstanding improvement in

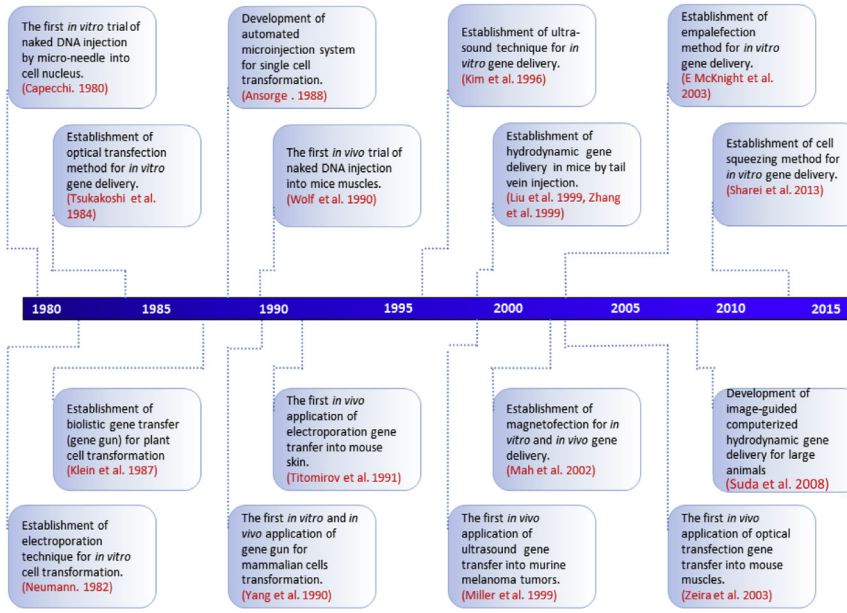

Figure 1 A timeline of the milestones in the development of physical methods for gene delivery.

the technique to include automated microinjection system controlling movement of micropipette and manipulator with precise cell positioning and injection times (Ansorge & Pepperkok, 1988). Computer-guided microinjection has also been developed to enhance reproducibility and minimize variation associated with manual operation (Pepperkok, Schneider, Philipson, & Ansorge, 1991). Such developments promoted microinjection as a typical procedure for single cell assays involving nuclear DNA transfer (Lamb, Gauthier-Rouviere, & Fernandez, 1996), and cytoplasmic, i.e., mitochondrial DNA transfer (Kagawa, Inoki, & Endo, 2001). To date, production of recombinant cell lines and transgenic animals remains the standard application of microinjection, because it is advantageous in bypassing cytoplasmic nucleases, and delivering precisely defined copy number of transgenes (Auerbach, 2004; Chenuet et al., 2009).

In vivo needle injection was also successfully demonstrated in mouse skeletal muscles and in other tissues, albeit low level of gene expression that was limited to the site of injection (Wolff et al., 1990). Although it has been suggested that the injected DNA is taken by cells through an active receptor-mediated process (Budker et al., 2000), DNA diffusion into cells through the membrane defects generated by needle insertion is more likely the mechanism as the gene expression was primarily located in the needle track. Although limited, the expression level of the transgene was sufficient in eliciting biological responses such as immune response, justifying the use of intramuscular injection of plasmid DNA as a vaccination strategy to express viral antigens (Danko & Wolff, 1994). Further success was demonstrated in gene therapy of ischemia in rabbits (Vincent et al., 2000), and in patients with limb ischemia (Kalka et al., 2000), both aimed to induce angiogenesis by increasing levels of vascular endothelial growth factor (VEGF) upon intramuscular injection of VEGF-coding plasmid DNA. Importantly, DNA transfer was not solely confined to muscular tissue, as evidenced by several studies involving direct injection of naked DNA into various tissues for different therapeutic purposes, such as liver (Hickman et al., 1994; Zhang et al., 1997), skin (Yu et al., 1999), lungs (Meyer, Thompson, Levy, Barron, & Szoka, 1995), and for intratumoral gene delivery (Yang & Huang, 1996).

A modified version of needle injection is impalefection, the use of nanofibers or nanowires instead of a needle to deliver genetic material into target cells. This method was established in 2003 using vertically aligned carbon nanofiber (VACNFs) array fixed on chips, and plasmid DNA adsorbed and tethered to VACNFs (McKnight, 2003). DNA-loaded VACNFs are integrated into cells by means of centrifugation of suspended cells into chips.

Following centrifugation and integration, chips are transferred into growth medium in culture dish to allow recovery and proliferation of transfected cells. This technique demonstrated promising transfection efficiency, while maintaining cell viability. Impalefection was later improved to increase the efficiency of transfection and to allow tracking of transgene expression spatially and temporally, along with viability assessment of transfected cells (McKnight et al., 2004). To date, impalefection has been explored for *in vitro* transfection, and further fabrication is clearly needed toward its *in vivo* applications. Though, the method holds the potential that was evident in several *in vitro* impalefection gene transfer studies (Mann et al., 2008; Pearce et al., 2013; Peckys, Melechko, Simpson, & McKnight, 2009). Presumably, *in vivo* application of impalefection would be utilizing chip arrays of optimized nanofibers with proper height and thickness that are pressed against target tissue or organ, forcing nanofiber penetration into target cells and release of surface-bound genetic material.

2.2 Gene Gun or "Biolistic" Gene Transfer

Gene gun, also called biolistic gene transfer, was first established in 1987 for plant cell transformation (Klein, Wolf, Wu, & Sanford, 1987), and later, it was successfully applied for gene transfer studies in mammalian cells *in vitro* as well as *in vivo* (Williams et al., 1991; Yang, Burkholder, Roberts, Martinell, & McCabe, 1990). Gene transfer is accomplished by bombarding target cells with DNA-coated gold particles driven by pressurized inert gas such as helium or by high-voltage electronic discharge. Efficient gene transfer necessitates fine optimization of the procedure to maintain penetration capacity, while minimizing tissue/cell damage. Among the parameters that impact the efficiency of gene transfer are the size and the density of microspheres, bombardment force, gene gun instrumentation, and microspheres to DNA ratio. Typically, these parameters vary with different types of cells and different tissues in animals (Eisenbraun, Fuller, & Haynes, 1993; Sanford, Smith, & Russell, 1993). Biolistic gene transfer is advantageous in being fast, simple, and highly efficient. Moreover, the technique is permissive to deliver wide range of macromolecules, such as nucleic acids and proteins. To date, DNA vaccination is the most common application of biolistic gene transfer because the technique efficiently delivers small amount of DNA sufficient to induce immune response against gene product, besides the immunogenicity of the technique itself; making the technique superior to other physical methods for DNA vaccination (Wang et al., 2008). Indeed, biolistic gene transfer has demonstrated great promise in preclinical models for DNA

vaccination, such as mice, rabbits, nonhuman primates, as well as in human clinical trials (Fuller, Loudon, & Schmaljohn, 2006). Aside from vaccination, the technique has been modestly applied in gene therapy trials in which a little amount of therapeutic protein is enough to elicit therapeutic response for cancer immunotherapy (Lin, Pulkkinen, Uitto, & Yoon, 2000; Seigne et al., 1999; Sun et al., 1995). Most applications of gene gun are limited to exposed tissues including skin and muscles. Though, with the aid of surgical procedures, inner organs were also targeted with gene gun such as liver (Kuriyama et al., 2000), neurons (McAllister, 2000), and brain (Zhang & Selzer, 2001). Despite the promising results in vaccination trials, the progress of biolistic gene transfer in clinics is challenged by limited efficiency to transfect larger and deeper areas, and the cost of the specialized gene gun and preparation of pure gold particles.

2.3 Electroporation

Electroporation-mediated gene transfer has been first and successfully established in 1982 by Neumann and collaborators (Neumann, Schaefer-Ridder, Wang, & Hofschneider, 1982), and since then, the technique has evolved as a powerful and widely used method of gene transfer that demonstrated prominent success and versatility in studies involved *in vitro* and *in vivo* gene delivery to various prokaryotic and eukaryotic cells. Historically, the concept of membrane permeation through the application of electrical impulses preceded studies of gene transfer, and was originally explored to understand membrane permeability to biological molecules such as catecholamines (Neumann & Rosenheck, 1972). The mechanism of permeation was revealed later to be transient pores created in the membranes, allowing large and/or ionic macromolecules like DNA, proteins, and even drugs to pass through (Chang & Reese, 1990). Importantly, these pores reseal within a few seconds to minutes, without significant impacts on membrane structure or cell viability (Weaver, 1995). The procedure of electroporation-mediated gene transfer comprises at least two electrodes connected to a power supply and the target cells are in between. *In vitro* electroporation is applied in a specialized cuvette having a suspension of cells and DNA and connected to a power supply, while the *in vivo* system involves electrodes inserted into and enclose the target tissue (Gehl, 2003). An electrical pulse is applied to cells, allowing DNA to get into cells. Transfection efficiency and reproducibility are controlled by tight adjustment of procedure parameters, such as the duration of pulse, frequency of electric shock, and the intensity of the electrical field. Gene transfer efficiency varies

significantly among different cell types and experimental conditions. For example, the use of dimethyl sulfoxide (DMSO) significantly enhanced the efficiency of electroporation and DNA uptake in mammalian cells (Melkonyan, Sorg, & Klempt, 1996). Moreover, several designs of electrodes have been developed such as plate electrodes, needle pair electrodes, needle array electrodes, and meander electrodes (Gilbert, Jaroszeski, & Heller, 1997; Tjelle, Salte, Mathiesen, & Kjeken, 2006; Zhang, Nolan, Kreitschitz, & Rabussay, 2002), each manipulates and tailors electrical field configuration toward the maximal efficiency of gene transfer and minimal tissue damage. Apart from technical features, *in vivo* electroporation for gene transfer started in the early 1990s (Titomirov, Sukharev, & Kistanova, 1991) even though the impressive outcomes came from clinical trials using electroporation to deliver chemotherapy drugs to tumors (Glass et al., 1996; Heller et al., 1996; Mir et al., 1991). The field of electroporation was then expanded to become among the most commonly used methods of gene transfer into various tissues and in different animal models. Skeletal muscles have been extensively utilized in gene transfer experiments using electroporation, as being easily accessible. Highly efficient platform for long duration of gene expression has been established for muscle-gene transfer comparing to other soft organs (Li & Benninger, 2002; McMahon & Wells, 2004). Certainly, different parameters were applied for optimal muscle transfection in murine (Tevz et al., 2008) versus large animals (Khan et al., 2003). The liver has also attained significant attention in gene therapy trials for liver diseases as well as other diseases, and indeed demonstrated great efficiency in transgene expression, largely due to its inherent function of protein synthesis, and highly vascularized structure. Strikingly, systemic administration of plasmid DNA via tail vein is superior to intrahepatic injection, resulting in more efficient gene transfer (Jaichandran et al., 2006). Electroporation-mediated gene transfer has been also assessed in other tissues and shown very encouraging results in pulmonary (Dean, Machado-Aranda, Blair-Parks, Yeldandi, & Young, 2003), renal (Tsujie, Isaka, Nakamura, Imai, & Hori, 2001), dermal (Gothelf & Gehl, 2010), cardiac (Harrison, Byrne, & Tung, 1998), pancreatic (Sato et al., 2013), corneal (Blair-Parks, Weston, & Dean, 2002), and intratumoral gene transfer (Heller & Heller, 2010). This prominent success advanced electroporation into gene therapy trials such as cancer gene therapy (Dolinsek et al., 2013; Sin et al., 2012), DNA vaccination (Ligtenberg, Rojas-Colonelli, Kiessling, & Lladser, 2013), gene therapy for liver cirrhosis (Kiyama et al., 2008) and hepatitis C infection (Weiland et al., 2013), renal failure (Brown, Bodles-Brakhop, Pope, & Draghia-Akli, 2009), and

ischemic diseases (Ouma et al., 2014). Similar to other methods of gene transfer, however, several challenges are yet to overcome, such as the collateral tissue damage, invasiveness, and the limited area of efficacy between electrodes, making it difficult to transfect large number of cells.

2.4 Hydrodynamic Gene Transfer "Hydroporation"

Hydrodynamic gene delivery was established in late-1990s (Liu, Song, & Liu, 1999; Zhang, Budker, & Wolff, 1999). It involves a rapid tail-vein injection of plasmid DNA into a mouse using a relatively large volume of DNA solution that induces transient cardiac congestion, resulting in an elevated hydrodynamic pressure in the inferior vena cava that drives DNA solution back to the liver and kidneys through hepatic and renal vein, respectively. Due to fenestrated endothelium in the liver, this pressure widens the pores of the liver fenestration and subsequently impacts hepatocytes surrounding the capillaries. It has been confirmed that hydrodynamic pressure-derived perforation or the so-called "hydroporation" of cell membrane and fluid entry is the underlying mechanism of gene transfer (Crespo et al., 2005; Zhang et al., 2004). Importantly, the pressure impact on the liver is transient and reversible, and hepatocytes recover functionally and structurally within 24–48 h post injection (Suda, Gao, Stolz, & Liu, 2007). The dynamic pressure is a function of the volume injected and the speed of injection, and thus, needs careful adjustment to ensure proper hydrodynamic pressure force that drives efficient gene transfer with minimal side effects. Certainly, these parameters vary with different anatomical features of the target organ, different structures of parenchymal cells, and different capillary types, i.e., fenestrated or continuous (Chen, Liu, & Lin, 2005; Danialou et al., 2005; Maruyama et al., 2002; Yoshino, Hashizume, & Kobayashi, 2006). Hydrodynamic gene transfer has been proven superior to the existing nonviral methods because it is highly efficient, relatively simple, safe, and versatile (Suda & Liu, 2007). Thus far, the liver is the primary organ targeted by hydrodynamic gene transfer, as being easily and noninvasively accessible via tail vein, as well as demonstrating highest levels of transgene expression among targeted organs (Song, Liu, Zhang, & Liu, 2002). Liver-directed hydrodynamic gene delivery is increasingly used with numerous applications, such as liver gene therapy and gene drug discovery, animal model establishment, and genetic studies of gene expression regulation (Bonamassa, Hai, & Liu, 2011). Gene transfer to other organs using hydrodynamics has also been explored, such as muscles that have shown comparable levels of transgene expression to liver in rodent and large animal

models (Hagstrom et al., 2004; Kamimura, Suda, Xu, Zhang, & Liu, 2009). In spite of similar underlying mechanism of gene transfer, technical aspects are quite different. DNA solution is typically applied into afferent or efferent vessels that are transiently occluded around the target tissue, and DNA solution is administered in the enclosed compartment. Hydrodynamic gene transfer has gained increased attention and becomes broadly applied in biomedical research to deliver DNA, RNA, proteins, and synthetic compounds to various tissues for different purposes. Indeed, it has been used in gene therapy studies for treatment of several diseases including growth hormone deficiency (Sondergaard, Dagnaes-Hansen, Flyvbjerg, & Jensen, 2003), hemophilia (Miao, 2005), diabetes (He et al., 2004; Vakili et al., 2013), obesity (Gao et al., 2013; Jiang, Yamato, & Miyazaki, 2003), hypertension (Romero-Vasquez et al., 2012), autoimmune myocarditis (Liu et al., 2005), muscular dystrophy (Zhang et al., 2010), renal ischemia (Hamar et al., 2004), DNA vaccination (Neal, Bates, Albertini, & Herweijer, 2007), and different types of cancers (Barnett et al., 2004; Maruyama et al., 2012; Wang, Chen, Tang, Zhang, & Hua, 2013; Wen, Matsumoto, Taniura, Tomioka, & Nakamura, 2004; Yazawa et al., 2006). In addition, the method was used to establish animal models for different diseases, such as mouse hepatitis B viral infection by transfecting hepatocytes *in vivo* with HBV genome expressing viral antigens and replicative intermediates, resulting in production of viral particles (Yang, Althage, Chung, & Chisari, 2002). Numerous modifications have been developed to adapt the technique of hydrodynamics for clinical and experimental needs. Recently, an automated injection device with computerized control has been developed for hydrodynamic gene transfer in large animals (Suda, Suda, & Liu, 2008). The device allows automated adjustment of injection parameters using intravascular pressure as a regulator. Further improvement was made in combining this device with an image-guided catheterization technique, allowing lobe-specific gene transfer to the liver of pigs (Kamimura et al., 2009), and offering great potential as the method of choice for clinical application in human gene therapy. Apart from gene transfer, the principle of hydrodynamics has been utilized for *in vivo* cell delivery as well. Hydrodynamic cell delivery was recently approved for establishment of the metastatic tumor model in mice, in which tumor cells are simultaneously seeded in liver, kidneys, and lungs (Li, Yao, & Liu, 2011). As being highly efficient and simple, hydrodynamic gene transfer truly boosts research progress in the field of gene therapy.

2.5 Ultrasound-Mediated Gene Transfer "Sonoporation"

Since the 1960s, ultrasound technique has been routinely used in clinical practice for various diagnostic and therapeutic purposes, and later in the 1990s, ultrasound was established as a method to enhance transdermal drug delivery (Bommannan, Okuyama, Stauffer, & Guy, 1992; Mitragotri, Edwards, Blankschtein, & Langer, 1995). Sonoporation was then successfully utilized to transfect fibroblast and chondrocytes with plasmid DNA using ultrasound waves applied through the walls of cell-culture plates and flasks (Kim, Greenleaf, Kinnick, Bronk, & Bolander, 1996). Albeit lesser effective than electroporation and hydrodynamic gene transfer, sonoporation has gained increased attention because it is advantageous for being simple, noninvasive, safe, and more tolerable than the other methods of gene transfer since no tissue damage is associated with application of ultrasound energy (Rychak & Klibanov, 2014). Similar to other physical methods, the underlying mechanism of sonoporation gene transfer is through transient permeation of cell membrane a principle called microbubble and cavitation. Upon application of ultrasound waves to cells in an *in vitro* or *in vivo* aqueous media, gas-filled and protein-stabilized bubbles are formed. The size of these bubbles is proportional to the applied energy. These bubbles oscillate in the ultrasound field and eventually collapse and release energy dramatically impacting and permeabilizing nearby cell membranes, and allowing macromolecules to get through transiently created pores (Cool, Geers, Lentacker, De Smedt, & Sanders, 2013; Wells, 2010). The overall efficiency of sonoporation varies in different experimental conditions, including the frequency of the applied ultrasound energy, the duration of treatment, plasmid DNA concentration, and even the ambient temperature. It also varies with different cells or tissue types. Sonoporation efficiency can be further improved by incorporation of echo-contrast agents that act as cavitation nuclei and facilitate energy transfer to increase permeabilization (Greenleaf, Bolander, Sarkar, Goldring, & Greenleaf, 1998; Miller, Pislaru, & Greenleaf, 2002). Therefore, these factors are carefully optimized toward specific tissues, models, and/or therapeutic needs (Pislaru et al., 2003). Sonoporation gene transfer has been investigated to transfect different tissues and demonstrated promising results, such as in muscles (Lu, Liang, Partridge, & Blomley, 2003), liver (Shen, Brayman, Chen, & Miao, 2008), lungs (Xenariou et al., 2007), heart (Fujii et al., 2011), vasculature (Taniyama et al., 2002), and solid tumors (Haag et al., 2006). Promising results were shown in gene therapy trials to treat liver fibrosis (Yang et al., 2013), myocardial

ischemia (Korpanty et al., 2005), diabetes (Chen et al., 2007), and different cancers (Fujii et al., 2013; Liao et al., 2012; Sakakima et al., 2005). Although the method is highly safe, the *in vivo* application of sonoporation is limited by the modest transfection efficiency in comparison to the other methods. It has been suggested that combined effect of sonoporation and other physical methods would enhance transfection efficiency. Indeed, combined sonoporation and electroporation to muscles showed transfection efficiency superior to either method alone (Yamashita et al., 2002).

2.6 Magnetofection

Magnetofection mediates gene transfer using supramagnetic iron oxide nanoparticles coated with DNA in presence of magnetic field. The principle of magnetism in targeted drug delivery has been applied since the early 1980s (Widder & Senyei, 1983), aiming to concentrate drug-loaded magnetic particles at the target site by means of magnetic field application. Magnetic targeting was later implemented in gene delivery (Mah et al., 2002), and thereafter, several systems have been developed where magnetic nanoparticles made of iron oxide are complexed to nonviral or viral vectors (Huth et al., 2004; Scherer et al., 2002), which greatly enhance gene transfer into target tissues. Mechanistically, magnetofection enhances gene delivery by guiding and maintaining DNA-loaded particles in close contact with target cells, and thus increasing cellular uptake of these particles through endocytosis. Further enhancement comes from magnetic field-facilitated extravasation of particles into surrounding tissue (Plank et al., 2003). Moreover, recent studies demonstrated that magnetic field efficiently increases cell membrane permeability by a yet unclear mechanism (Shankayi, Firoozabadi, Mansourian, & Mahna, 2014). Magnetofection has been broadly used in gene transfer for cultured cells, such as cultured endothelial cells (Krotz, Sohn, Gloe, Plank, & Pohl, 2003), and to a lesser extent, for *in vivo* gene transfer. The method has been successfully employed for intratumoral delivery of anti-metastatic NM23-H1 gene that results in suppression of pulmonary metastasis in mouse model (Li et al., 2009). It has also been utilized in intradermal gene delivery of VEGF to induce angiogenesis and perfusion in ischemic skin flaps model in rats (Holzbach et al., 2010). Several challenges remain for *in vivo* magnetofection, such as inferior transfection efficiency, rapid systemic clearance of iron oxide particles, and increased safety concern regarding the accumulation of iron oxide in cells, especially with multidosing experiments.

2.7 Laser-Mediated Gene Transfer "Optical Transfection"

Laser irradiation is another form of physical force that has been explored to permeabilize cell membrane in order to facilitate gene transfer. Laser-mediated gene transfer, also called optoporation, has been established in 1980s (Kurata, Tsukakoshi, Kasuya, & Ikawa, 1986; Tsukakoshi, Kurata, Nomiya, Ikawa, & Kasuya, 1984), allowing genetic material in culture media to get into cells. Several advancements have been achieved in the field of optical transfection, allowing selective targeting of single cell, or even a particular subcellular structure, such as nuclei and mitochondria, using various wavelengths and power densities (Yao, Zhang, Rahmanzadeh, & Huettmann, 2008). While Neodymium-doped yttrium aluminum garnet (Nd:YAG) was used originally as a laser source, several sources have later been developed such as argon ion (Palumbo et al., 1996), holmium YAG (Ho:YAG) (Sagi et al., 2003), and titanium sapphire (Zeira et al., 2003). Laser-beam intensity is controlled by pulse generator and focused on the target area by a lens. Optical transfection is advantageous in being safe and noninvasive. Optical transfection has demonstrated promising success in various *in vitro* studies (Stevenson, Gunn-Moore, Campbell, & Dholakia, 2010), but it is still a subject of research to be applied *in vivo*. This is largely due to the relatively high cost and the limited efficiency because of limited penetrating capacity (approximately 2 mm) to deeper tissues as well as limited impacted area. Therefore, *in vivo* application was limited to exposed regions such as muscles (Zeira et al., 2003), skin (Zeira et al., 2007), and for intratumor gene transfer (Tsen et al., 2009).

2.8 Cell Squeezing "Microfluidics Gene Transfer"

Microfluidic gene transfer has been newly developed for *in vitro* delivery of proteins and nucleic acids into cells using the principle of cell deformation (Sharei et al., 2013). The original concept emerged in early 1990s in studies of macromolecules loading into cells using a method that was developed back then called syringe loading (Clarke & McNeil, 1992). It was shown that the mechanism of transfer is the transient defects in cell membrane that are created as cells are passed back and forth through a standard syringe needle or similar narrow orifice. The principle, also called shear-induced intracellular uptake, was later refined and special devices were developed in which cells are deformed and the membrane is perforated upon flow through microchannels with proper velocity. The method indeed successfully mediated uptake of large molecules such as dextran and bovine serum

albumin into suspended prostate cancer cells, without compromising cell viability (Hallow et al., 2008). Recently, this method was implemented in gene transfer and demonstrated promising success for *in vitro* siRNA delivery (Sharei et al., 2013). It has been shown that the size and the frequency of the transient holes, and hence transfection efficiency are functions of the shear and compressive forces that the cells experience when passing through the constriction. In turn, these forces are determined by the flow rate of cells, dimensions, and the number of constrictions. More recently, kinetic studies of membrane recovery after cell squeezing technique have shown that calcium content in cell medium is another major determinant of transfection efficiency, and that calcium depletion significantly increased macromolecule uptake by squeezed cells (Sharei et al., 2014). This is because the active membrane recovery is mediated by calcium-dependent signaling (McNeil & Steinhardt, 2003).

3. FUTURE PERSPECTIVES

Revolutionary advances have taken place in the field of gene delivery over the past decades. Physical methods, in particular, have gained increased attention in attempt to overcome barriers of chemical and viral methods. However, clinical application of physical methods is rather limited, largely imposed by some challenges that necessitate additional research and improvements to the current systems. The hallmark for efficient gene transfer is the application of mechanical forces to disrupt cell membranes for the DNA to get in. These forces, however, are not risk free, as in many cases efficient gene transfer is accompanied with local tissue damage, which is often considered as intolerable in clinical practice. In addition, while skin is easily accessible, gene transfer to inner organs, such as liver and kidneys, with physical methods requires invasive, usually surgical procedures to insert the applicators close to the target tissues, presenting an additional medical concern for disease management. Moreover, the tissue fraction that is successfully transfected with most physical methods is often limited. This might explain the bias in exploring physical methods in gene therapy using secreted proteins more than intracellular proteins, where the locally expressed protein is sufficient to drive global effects, such as vaccination and growth-factor replacement therapies.

Nonetheless, the technology of physics-based gene delivery has now advanced to a point from where it seems feasible to implement minimally invasive strategies for gene transfer into various target tissues, with adequate

levels of transfection and minimal side effects. This is clearly illustrated in the development of image-guided computerized hydrodynamic gene transfer into large animals. The method combines tunable parameters with facile application into a specific organ, and specific area within the organ, and thus, it truly holds the promise for clinical applications in human gene therapy. Moreover, these computerized methods will also enhance the reproducibility and reduce variations commonly associated with manual procedures. Future research will focus on the amenability to physically manipulate biological and physiological features of tissues aiming to facilitate gene transfer. Joint application of different kinds of physical forces will also be considered sincerely to improve the overall efficiency, and to minimize the magnitude of the applied forces. Innovative integration of physics, chemistry, biology, and computer engineering will pave the way for the urgently needed breakthroughs in the field of nonviral gene delivery and for human gene therapy.

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CHAPTER TWO

Nonviral Gene Delivery Systems by the Combination of Bubble Liposomes and Ultrasound

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Contents

Abstract

The combination of therapeutic ultrasound (US) and nano/microbubbles is an important system for establishing a novel and noninvasive gene delivery system. Genes are delivered more efficiently using this system compared with a conventional nonviral vector system such as the lipofection method, resulting in higher gene expression. This higher efficiency is due to the gene being delivered into the cytosol and bypassing the endocytosis pathway. Many *in vivo* studies have demonstrated US-mediated gene delivery with nano/microbubbles, and several gene therapy feasibility studies for various diseases have been reported. In addition, nano/microbubbles can deliver genes site specifically by the control of US exposure site. In the present review, we summarize

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the gene delivery systems by the combination of nano/microbubbles and US, describe their properties, and assess applications and challenges of US theranostics.

1. INTRODUCTION

Gene therapy has potential for treating cancer and genetic diseases. Viral vectors are investigated and shown effective for gene transduction, but some problems have become evident (Check, 2002, 2003; Marshall, 1999). Delivery vectors that are highly efficient for gene transduction must also be safe and easy to use. Nonviral vectors have recently received attention as gene carriers, but their transduction efficiency is very low, although efforts have been made to address this (Itaka et al., 2007; Kogure, Akita, Yamada, & Harashima, 2008; Liu, Conwell, Yuan, Shollenberger, & Huang, 2007). Toward this end, ultrasound (US) has been investigated for improving the efficiency of transgene delivery and holds promise as a means of generating a noninvasive gene delivery system.

Sonoporation improves the efficiency of gene delivery into tissues and cells by perturbing the cell membrane and causing transient pores to open in the membrane, thus facilitating gene entry into the cell (Fechheimer et al., 1987; Miller, Miller, & Brayman, 1996). In addition, it has been reported that microbubbles, used as US contrast agents play an important role in enhancing the efficiency of gene delivery without causing cell damage (Greenleaf, Bolander, Sarkar, Goldring, & Greenleaf, 1998). In general, cell damage is dependent on the US intensity, the exposure time, the concentration of microbubbles, and the cell type, with US intensity and exposure time being particularly important. Therefore, effective US-mediated gene delivery requires the optimization of the US exposure conditions (Feril, Ogawa, Tachibana, & Kopndo, 2006; Li, Tachibana, Kuroki, & Kuroki, 2003; Pislaru et al., 2003; Suzuki, Takizawa, Negishi, et al., 2008). Some researchers studied about the cell damage by the disruption of microbubbles with US exposure (Feril et al., 2003; Guo et al., 2006; Hassan et al., 2009, 2010; Kudo, Okada, & Yamamoto, 2009; Wells, 2010). These reports provide important information for US-mediated gene delivery utilizing microbubbles.

Microbubbles are destroyed by exposure to US, generating microstreams or microjets which result in shear stress to cells and the generation of transient holes in the cell membrane (Taniyama et al., 2002). Since this approach can be used to deliver extracellular material such as genes into cells, microbubbles could facilitate US-mediated gene delivery. In addition, submicron-sized

bubbles (nanobubbles), which are smaller than conventional microbubbles, were recently reported (Gao, Kennedy, Christensen, & Rapoport, 2008; Wang, Li, Zhou, Huang, & Xu, 2010), and we have developed novel liposomal nanobubbles (bubble liposomes (BLs)) (Negishi et al., 2008; Suzuki et al., 2009, 2010; Suzuki & Maruyama, 2010; Suzuki, Takizawa, Kuwata, et al., 2008; Suzuki, Takizawa, Negishi, et al., 2008; Suzuki, Takizawa, Negishi, Hagisawa, et al., 2007; Suzuki, Takizawa, Negishi, Utoguchi, et al., 2007; Un et al., 2010; Yamashita et al., 2007). These nanobubbles can also be used to enhance the efficiency of US-mediated gene delivery. In this review, we describe US-mediated delivery systems combined with nano/microbubbles and discuss their feasibility as nonviral vector systems.

2. MICROBUBBLES AND ULTRASOUND

The behavior of microbubbles depends on the amplitude of the US used. Very low acoustic pressure (mechanical index (MI) < 0.05–0.1) induces linear oscillation of the microbubble, and the reflected frequency is equal to the transmitted frequency (Figure 1(A)). An increase in acoustic pressure ($0.1 < \text{MI} < 0.3$), referred to as low-power imaging, causes nonlinear expansion and compression of the microbubble (Figure 1(B)). The bubble is somewhat more resistant to compression than to expansion, a phenomenon known as stable or noninertial cavitation, resulting in the emission of nonlinear harmonic signals at multiples of the transmitted frequency (Unger, Hersh, Vannan, Matsunaga, & McCreery, 2001). Harmonic imaging with microbubbles enhances the bubble-to-tissue backscatter signal ratio due to insignificant harmonic backscatter from tissue in this range of MI. Therefore, this technique can improve the signal-noise ratio and be useful in left ventricular pacification imaging (Mulvagh et al., 2000). In addition, stable or noninertial cavitation can enhance transient cell membrane permeability (Figure 2(A)) (van Wamel et al., 2006). Machluf et al. reported that the exposure of cells to US (0.16 MI, 1 MHz) in the presence of microbubbles resulted in the delivery of plasmid DNA (pDNA) into the cells (Duvshani-Eshet, Adam, et al., 2006; Duvshani-Eshet, Baruch, et al., 2006).

Higher acoustic pressure ($\text{MI} > 0.3$–0.6) causes forced expansion and compression of the microbubble and results in bubble disruption (collapse) (Figure 1(C)). Bubble disruption by this inertial cavitation is utilized as flash-replenishment in reperfusion study of diagnosis (Kalantarinia, Belcik, Patrie, & Wei, 2009). This inertial cavitation induces microstreams/microjets

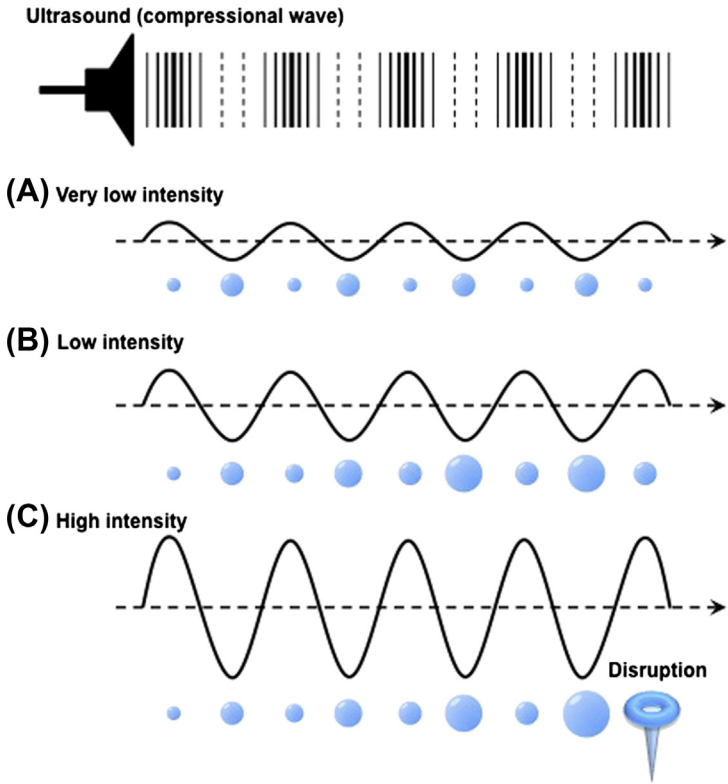

Figure 1 ***Schematics showing microbubble behavior in acoustic fields***. (A) Very low intensity ultrasound (US) induces the linear oscillation of the microbubble. (B) Low intensity US induces the oscillation of the microbubble, with a gradual increase in the diameter of the microbubble. Stable oscillation occurs when the microbubble reaches its resonant diameter. (C) High intensity US causes a rapid increase in the diameter of the microbubble for a few cycles, which induces bubble disruption.

around the bubbles. These microstreams/microjets can enhance the permeability of the cell membrane due to the formation of transient pores (Figure 2(B)) (Taniyama et al., 2002). In the presence of nano-/microbubbles, the threshold for cavitation decreases, allowing the destruction of the microbubbles at lower US energies.

3. BUBBLE LIPOSOMES

As mentioned above, microbubbles are contrast agents used in US imaging. Microbubbles can also be used to improve transfection efficiency

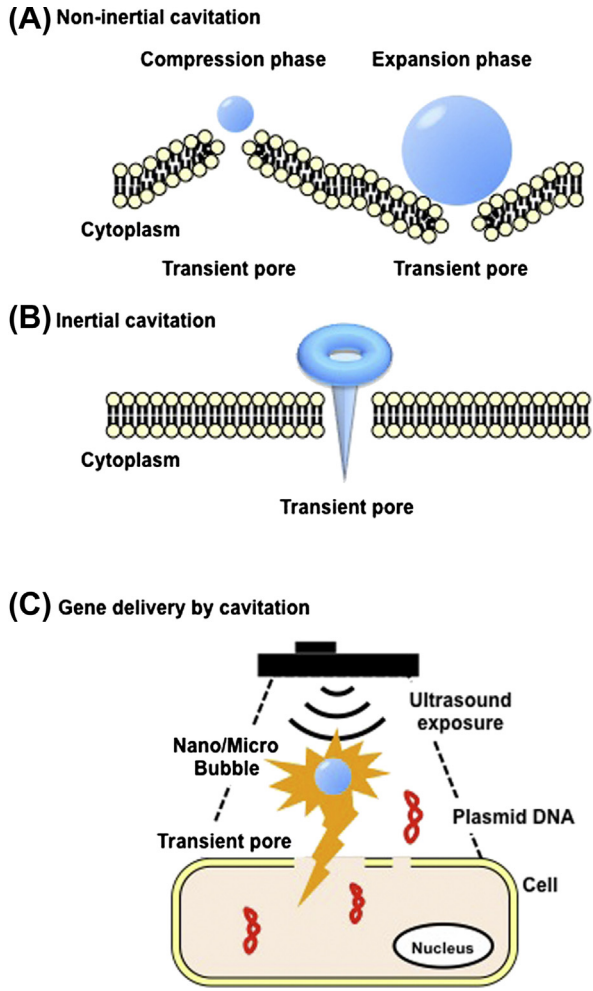

Figure 2 ***Schematics showing pore formation in the cell membrane by oscillating or disrupting microbubbles.*** (A) The pushing and pulling behavior (noninertial cavitation) of microbubbles and (B) the collapse of microbubbles (inertial cavitation) rupturing the cell membrane and creating pores allowing *trans*-membrane flux of fluid and macromolecules such as plasmid DNA and oligonucleotides (C).

when combined with US. Microbubbles are generally unstable and have a mean diameter of between 1 and 6 μm, making them too large for intravascular applications (Lindner, 2004). Moreover, it is difficult to modify their surface with functional molecules. Therefore, microbubbles should be small and stable, and their surface should be easily modified with functional molecules for targeting.

Liposomes have several advantages as drug, antigen and gene delivery carriers as their size can be easily controlled, and they can be modified with targeting molecules. Therefore, we used liposome technology to develop novel BLs containing the US gas, perfluoropropane (Figure 3(A)). BLs are about 500 nm in diameter, making them smaller than Sonazoid which is US contrast agent (about 2 μm) (Figure 3(B)). We also confirmed the structures of BLs by transmission electron microscopy and observed nanobubbles in the lipid bilayer (Figure 3(C)). Another morphological studies of BLs using transmission electron microscopy by Kodama et al. (2010) showed that gas and liquid seemed to be encapsulated together by a single lipid bilayer.

4. *IN VITRO* pDNA DELIVERY WITH BLs

Gene therapy requires highly efficient and safe gene delivery vectors. We recently developed polyethylenglycol-modified liposomes containing US imaging gas and demonstrated that these BLs act as US imaging agents, and induce cavitation with US exposure (Suzuki, Takizawa, Negishi, Hagisawa, et al., 2007). We investigated *in vitro* whether the combination

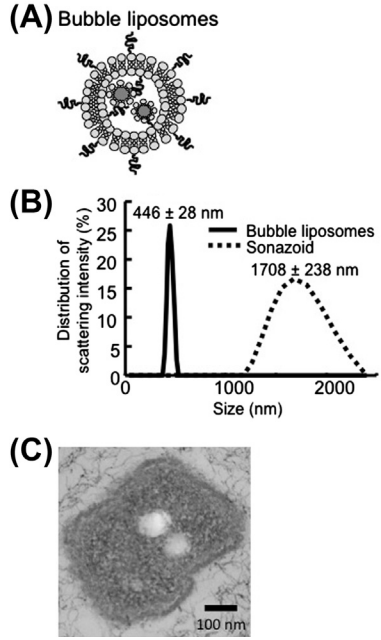

Figure 3 ***BLs***. (A) Schematic of a BL. (B) Size distribution of BLs and Sonazoid. (C) Transmission electron microscopic image of a BL.

of BLs and US could provide a gene delivery tool. First, we attempted to transfect pDNA encoding firefly luciferase (pCMV-Luc) into COS-7 with BLs and US. Luciferase activity in COS-7 cells treated with BLs and US was higher than in cells treated with BLs or US alone. Next, we demonstrated that BLs and US could be used to transfect pDNA into various cell lines such as S-180, Colon 26, B16BL6, Jurkat cells derived from T-cell lines, and human umbilical vein endothelial cells (Figure 4). Since it is difficult to transfect lymphocytes with pDNA using nonviral vectors, the combination of BLs and US holds promise as tools for gene transfection *in vitro*.

5. *IN VIVO* pDNA DELIVERY WITH BLs

Cancer gene therapy requires the delivery of genes into tumor tissue with high efficiency, safety, and minimal invasiveness. We attempted to deliver pDNA into tumor tissue using BLs and US. B6C3F1 tumor model mice were intradermally inoculated with Murine ovarian carcinoma (OV-HM) cells in the flank. After 7 days, a mixture of BLs (2.5 μg of lipid) and pCMV-Luc (10 μg) was injected into the tumor, and US (1 MHz, 0.7 W/cm^2, 1 min) was transdermally applied to the tumor tissue. Transfection efficiency using a conventional lipofection method was also investigated. The complexes of Lipofectamine 2000 (20 μg) and pCMV-Luc (10 μg) were injected into the tumors. Luciferase activity was measured 2 days after BLs with US or conventional transfection. Tumors treated with BLs and US showed higher luciferase activity compared with tumors

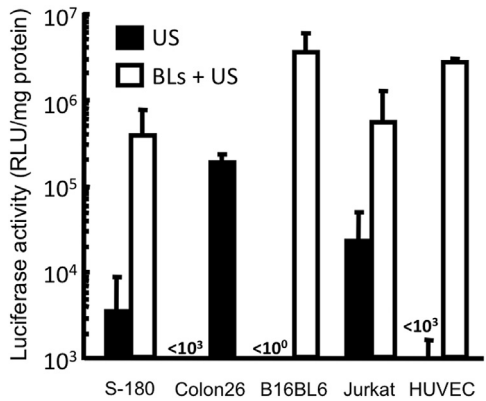

Figure 4 ***pDNA delivery with BLs and US* in vitro.** Luciferase activities in various types of cells transfected with BLs and US. BL: Bubble liposomes, US: Ultrasound.

treated with BLs or US alone, or with conventional lipofection. This result indicates that BLs and US can efficiently deliver pDNA into tumor tissue *in vivo*, and motivated us to examine whether BLs and US could be used for cancer gene therapy. Interleukin-12 (IL-12) exhibits immunomodulatory antitumor effects and is considered an effective antitumor agent (Brunda, 1994; Nastala et al., 1994), but its short half-life and systemic toxicity following intravenous injection are major obstacles to its therapeutic use (Atkins et al., 1997; Colombo et al., 1996). Therefore, we transfected pDNA encoding the IL-12 gene (pCMV-IL-12) into tumor tissue using BLs and US with the aim of achieving high local expression of IL-12. When pCMV-IL-12 was transfected using BLs, US, or Lipofectamine 2000, tumor growth was not suppressed. In contrast, transfection of pCMV-IL-12 with BLs and US suppressed tumor growth significantly (Figure 5). To investigate the mechanism behind the anti-tumor effects of pCMV-IL-12 transfected using BLs and US, we assessed the involvement of $CD4^+$ and $CD8^+$ T cells and natural killer (NK) cells. The depletion of $CD8^+$ T cells effectively blocked the anti-tumor effect of pCMV-IL-12 transfected using BLs and US. These results suggest that the combination of BLs and US can effectively induce sufficient IL-12 expression to cause anti-tumor immune responses.

Systemic gene delivery techniques are ideal for cancer therapy because they can deliver genes to tumors deep in the body. We confirmed that BLs act as US imaging agents for several minutes following intravascular

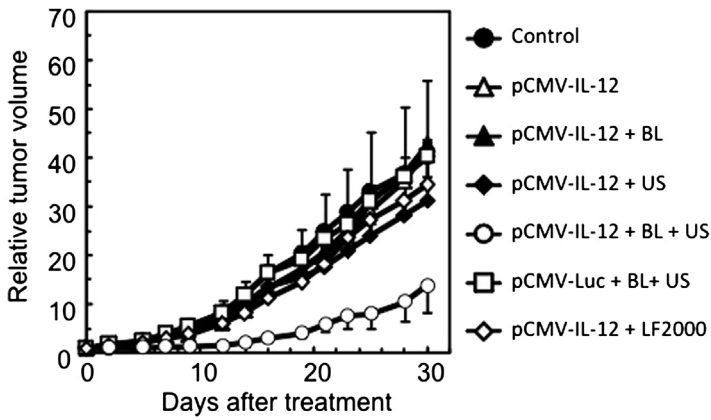

Figure 5 ***Antitumor effect of IL-12 gene delivery with BLs and US***. Antitumor effect was evaluated by measuring tumor volume. BL: Bubble liposomes, US: Ultrasound, LF2000: Lipofectamine 2000, pCMV-IL-12: pDNA encoding the IL-12 gene, pCMV-Luc: pDNA encoding the Luciferase gene.

administration. We attempted to deliver genes into tumor tissue using BLs and US by intravascular administration. S-180 cells were inoculated into the left footpad of ddY mice; after 4 days, 100 μl of pCMV-Luc (10 μg) and BLs (100 μg) were injected into the femoral artery and US (0.7 MHz, 1.2 W/cm^2, 2 min) was immediately applied transdermally to the tumor tissue. Transfection with BLs, US, or Lipofectamine 2000 alone was also conducted. Two days following transfection, the luciferase activity of the tumor tissues was measured. The luciferase activity of tumors treated with BLs and US was higher than that of tumors treated with BLs, US, or Lipofectamine 2000, suggesting that BLs and US can efficiently deliver genes into tumor tissue following intravascular administration. Thus, BLs and US hold promise for cancer gene delivery by local or intravenous administration.

6. *IN VITRO/IN VIVO* SMALL INTERFERING RNA DELIVERY WITH BLs

Small interfering RNA (siRNA) is another potential therapeutic tool, but its clinical application has been hindered by the lack of efficient and robust delivery systems. We investigated whether the combination of BLs and US could be used to deliver siRNA *in vitro* and *in vivo* (Negishi et al., 2008). We first tried to transfect pDNA and siRNA into COS-7 cells. Cells were cotransfected with pCMV-GL3 together with a nontargeting control or luciferase-targeting siRNA (siCont or siGL3) at concentrations between 15 and 50 nM. The expression of luciferase in cells was efficiently inhibited in a siRNA dose-dependent manner. The downregulation of luciferase activity was also observed in the presence of a high serum concentration. This is consistent with our previous data showing pDNA delivery using BLs and US (Suzuki, Takizawa, Negishi, et al., 2008). These results might indicate that siRNA is delivered into the cytoplasm quickly and directly following transfection. Indeed, fluorescently labeled siRNA was homogeneously localized primarily in the cytoplasm shortly after transfection (Figure 6). The intracellular localization of pDNA transfected with BLs and US also showed the same tendency. These results differ from an observation made regarding the endosomal pathway of the lipoplex. Furthermore, the gene-silencing efficiency of transfected siRNA using BLs and US was independent of the culture temperature (4 °C or 37 °C) and the presence of chloroquine, which is recognized as an endosomolytic agent. Thus, endocytosis participates minimally in the transfection process using BLs and US, and siRNA is directly and instantaneously delivered into the

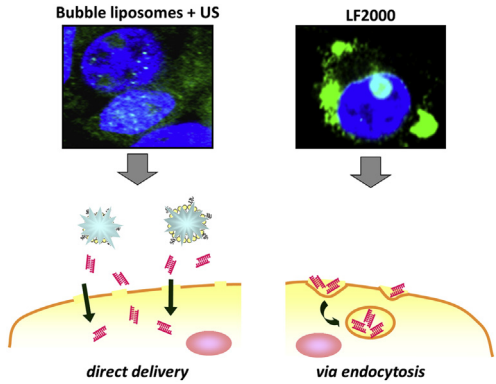

Figure 6 ***Mechanism of action of bubble liposomes (BLs) and ultrasound (US).*** Localization of FITC-siRNA after transfection using BLs and US, or Lipofectamine2000 (LF2000), and differences in their mechanism of action. *Reprinted from Negishi et al. (2008) with permission.* (For interpretation of the references to color in this figure see the color plate.)

cytoplasm. Recent reports show that siRNAs can activate the innate immunity in mammalian cells, and that the immune response is mainly due to Toll-like receptors (TLRs) in the endosome (Elbashir et al., 2001; Sioud, 2007; Sledz, Holko, de Veer, Silverman, & Williams, 2003). Transfection using BLs and US may bypass the activation of an immune response via TLRs; if so, direct delivery of siRNA into the cytoplasm of target cells using a combination of BLs and US may be a promising siRNA delivery system.

When BLs and US were used to cotransfect pDNA encoding luciferase and its siRNA into the muscles of mice, luciferase expression was suppressed for up to 7 days, further indicating that BLs could be a useful tool *in vivo*. Furthermore, the use of BLs and US to transfect siRNA targeting an endogenous gene (GSK-3β) into muscle suppressed GSK-3β protein expression in the US-exposed area, resulting in a gene-silencing effect in the muscle. We also attempted to transfect pDNA and siRNA into skin and kidney. As a result, gene-silencing effect was observed in the skin. In contrast, in kidney, gene-silencing effect was not observed and the luciferase expression was not even observed in the group not transfected with siRNA. These results were assumed to be due to the direct injection into the parenchymal tissue of kidney, the excretory organ of pDNA and siRNA (Kobayashi, Kuramoto, Yamaoka, Hashida, & Takakura, 2001; Lu, Xie, & Woodle, 2005). Therefore, by the application of intravascular administration or other methods suited for target tissue, BLs and US might be a useful tool for the delivery of siRNA into widespread tissue (Chen et al., 2006).

7. siRNA-LOADED BLs

BLs are composed of neutral lipids, making it difficult to colocalize BLs in blood vessels after intravenous administration. In addition, siRNA delivered via systemic injection is prone to nuclease degradation, interaction with plasma proteins, and rapid removal from circulation, leading to a reduction in transfection efficiency *in vivo*. To address these issues, we tested two methods of loading siRNA to BLs in an attempt to make a more effective delivery tool for systemic injections (Figure 7).

7.1 Cholesterol-Conjugated siRNA-Loaded BLs

Cholesterol fits its nonpolar part between alkyl chains of phospholipids and is appropriate for incorporation into the phospholipid bilayer because of its easy-to-fit structure and its physical influence on membrane properties such as fluidity and permeability. Thus, cholesterol is often incorporated into liposomes used as drug carriers. Cholesterol-conjugated siRNA (chol-siRNA) was used to embed siRNA into BLs (Negishi et al., 2011). Liposomes-containing chol-siRNA were prepared by a reverse-phase evaporation method. To further prepare chol-siRNA-loaded BLs (chol-si-BLs), liposome suspensions were filled with perfluoropropane gas, capped, and then pressured with perfluoropropane gas. The particle size of the chol-si-BLs was approximately 500 nm and the zeta potential was neutral. In addition, like conventional BLs without cholesterol, chol-si-BLs could be used as US

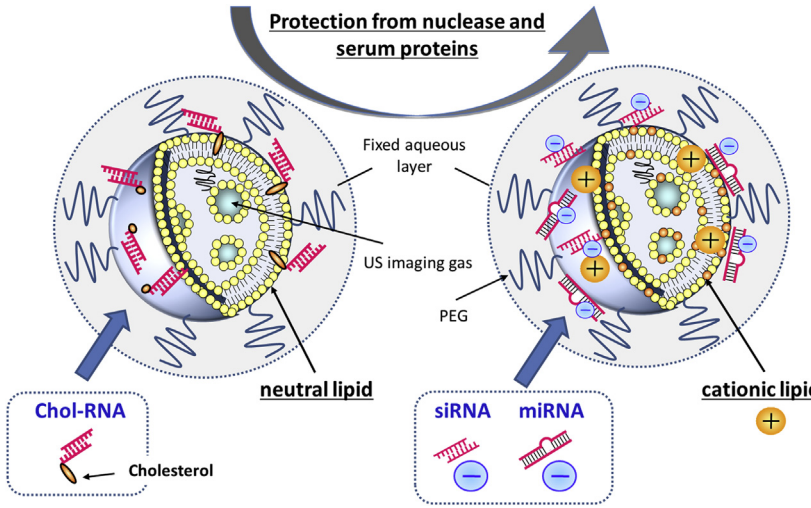

Figure 7 ***Schematic showing a nucleic acid-loaded bubble liposomes (BL).*** (For interpretation of the references to color in this figure see the color plate.)

contrast agents. To confirm whether siRNA could be loaded into the chol-BLs, we used Fluorescein isothiocyanate (FITC)-labeled chol-siRNA (chol-FITC-siRNA) and a fluorescence-activated cell sorter, FACSCanto. Up to 0.4 mol% chol-siRNA (of the total lipids) could be loaded into BLs. This amount of siRNA was more than six times compared to siRNA that showed approximately 80% gene silencing against luciferase gene expression by pDNA cotransfected *in vitro*. The gene-silencing effect of chol-si-BLs combined with US suggested that exposure to US-induced cavitation of the chol-si-BLs, the release of siRNA, and the delivery of siRNA into the cytoplasm within a fairly short time. Chol-siRNA apparently embeds easily in the lipid membrane and so may not be easily released from the lipid bilayer. The amount of chol-siRNA transfected into cells might be significantly increased if its release rate from lipids by US exposure could be increased. Furthermore, it was found that the degradation of the siRNA could be avoided, even if the chol-si-BLs were exposed to RNase. The chol-siRNA embedded in the outside lipid layer of BLs seemed susceptible to degradation by nuclease. It has been reported that the modification of short and long polyethylene glycol (PEG) chains for liposome preparations affects the thickness of the PEG-water interfacial layer (fixed aqueous layer; Figure 7) (Sadzuka et al., 2002). BLs with modified short and long PEG chains might stabilize siRNA. Using colon26-Luc cells stably expressing firefly luciferase and luciferase siRNA, approximately 50% luciferase expression was specifically blocked following transfection with chol-si-BLs and US. It was recently demonstrated that chol-siRNA can improve pharmacokinetic and cellular uptake of siRNA in mice after a systemic injection (Soutschek et al., 2004). Chol-siRNA apparently incorporated into lipoprotein particles and was easily internalized through receptor-mediated processes (Wolfrum et al., 2007). Although chol-siRNA alone seems to be delivered to a limited set of tissues, such as the liver, adrenal gland, and kidney, the combination of chol-si-BLs and transdermal US exposure may permit site-specific siRNA delivery to other tissues.

7.2 siRNA-Loaded BLs Using Electrostatic Interaction between Cationic Lipid and siRNA

Cationic lipids are useful for loading siRNA into BLs because the lipids can interact with negatively charged siRNA. We initially prepared BLs comprising 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a cationic lipid often used for gene delivery (Endo-Takahashi et al., 2012). The BLs were composed of 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine, DOTAP, DSPE-PEG_{2000}, and DSPE-PEG_{750} in a 79:15:3:3 M ratio.

Using a high-frequency US imaging system, we confirmed that BLs with this composition functioned as an echo-contrast agent (Negishi et al., 2012). Flow cytometry confirmed that siRNA could interact with these BLs, that the interaction was due to the cationic charge on DOTAP, and that BLs containing DOTAP, PEG_{750}, and PEG_{2000} could be loaded with much more siRNA compared with BLs containing DOTAP and PEG_{2000}. We believe that structural changes in PEG caused by the short and long chains facilitated interaction between the cationic lipid and anionic siRNA. These results suggested that siRNA could be loaded into the BLs both by electrostatic interactions and the fixed aqueous layer entrapped by PEG. We also showed that siRNA electrostatically bound to DOTAP-containing BLs exhibit increased stability in 50% serum compared with free siRNA. We next attempted to transfect siRNA into cells previously transfected with the luciferase gene using si-BLs and US exposure. The gene-silencing effect of siRNA was comparable with that of siRNA transfected using conventional BLs and US. Therefore, it appears that the exposure to US-induced cavitation, the release of siRNA from the BLs, and the delivery of siRNA into the cytoplasm. The siRNA was electrostatically bound to only the outside of BLs containing cationic lipids, possibly resulting in a smaller amount of siRNA available for delivery compared to chol-si-BLs. However, si-BLs are easier to prepare than chol-si-BLs, and the carrier BLs do not need to be reprepared every time the siRNA is changed. For these reasons, BLs containing DOTAP could be a convenient siRNA carrier. Furthermore, BLs containing cationic lipid are expected to have widespread application for the delivery of various negatively charged molecules. Indeed, we showed in *in vitro* and *in vivo* studies that DOTAP-containing BLs are useful for delivering pDNA (Endo-Takahashi et al., 2012; Negishi et al., 2012). Short-chain and unsaturated fatty acids increase membrane fluidity (Lian & Ho, 2001). DOTAP is an unsaturated fatty acid believed to destabilize the BL membrane. Indeed, increased DOTAP content hinders gas entrapment (Endo-Takahashi et al., 2012). We therefore expected that BLs containing saturated cationic lipids would improve the stability of the liposomal membrane. As expected, BLs containing three types of saturated cationic lipids (1,2-stearoyl-3-trimethylammonium-propane (DSTAP), 1,2-distearoyl-3-dimethylammonium-propane (DSDAP), dimethyldioctadecylammonium bromide (DDAB)) were more effective than BLs containing DOTAP as a contrast agent (Endo-Takahashi et al., 2013), with BLs containing DSDAP proving especially effective for US imaging and transfection. These results suggested that changing the cationic lipid stabilized the membrane and improved gas retention.

As discussed above, BLs combined with US are effective tools for gene delivery if injected locally. However, siRNA can be transfected into cells using novel and efficient siRNA carriers such as chol-si-BLs or si-BLs. For example, the systemic injection of chol-si-BLs or si-BL into kidney silenced genes that were not silenced following local injection, and allowed gene silencing in deep tissues that are difficult to inject directly.

Taken together, it is expected that tissue- or organ-specific delivery of siRNA, followed by a specific gene-silencing effect, may be achieved by the technologies of BLs combined with targeted US exposure, leading to clinical applications for various diseases.

8. MICRORNA-LOADED BLs

MicroRNAs (miRNAs) are involved in biological functions, and their dysregulation often leads to human diseases (McManus & Sharp, 2002). Recently, a large number of miRNAs were identified as key targets for therapeutic intervention. However, a vehicle for efficiently delivering therapeutic RNA to its target tissue is required for miRNA-based therapeutics (Zhang, Wang, & Gemeinhart, 2013). We therefore attempted to prepare miRNA-loaded BLs (mi-BLs) and evaluated their utility using a hindlimb ischemia model and miR-126 (Endo-Takahashi et al., 2014), which promotes angiogenesis by canceling the negative regulators of vascular endothelial growth factor (VEGF) signaling (Fish et al., 2008; van Solingen et al., 2009; Wang et al., 2008). Before the *in vivo* transfection experiments, we confirmed the interaction between miRNA and BLs containing a cationic lipid previously reported effective for the preparation of pDNA loaded-BLs (Endo-Takahashi et al., 2013). The amount of miRNA bound to the BLs increased in the presence of cationic lipid. Next, mi-BLs were delivered to a hindlimb ischemia mouse model via intravascular injection. mi-BLs at the ischemic site were visualized by diagnostic US, and delivered miR-126 following therapeutic US. The delivered miR-126 increased angiogenic gene expression and improved blood flow (Figure 8). Thus, the combination of mi-BLs and US exposure may serve as a theranostic agent with combined diagnostic and therapeutic properties (Endo-Takahashi et al., 2014).

9. ENDOSOMAL ESCAPE ENHANCED BY BLs

The importance of intracellular trafficking of gene delivery vectors has been emphasized. To achieve efficient gene transfection, vectors must be

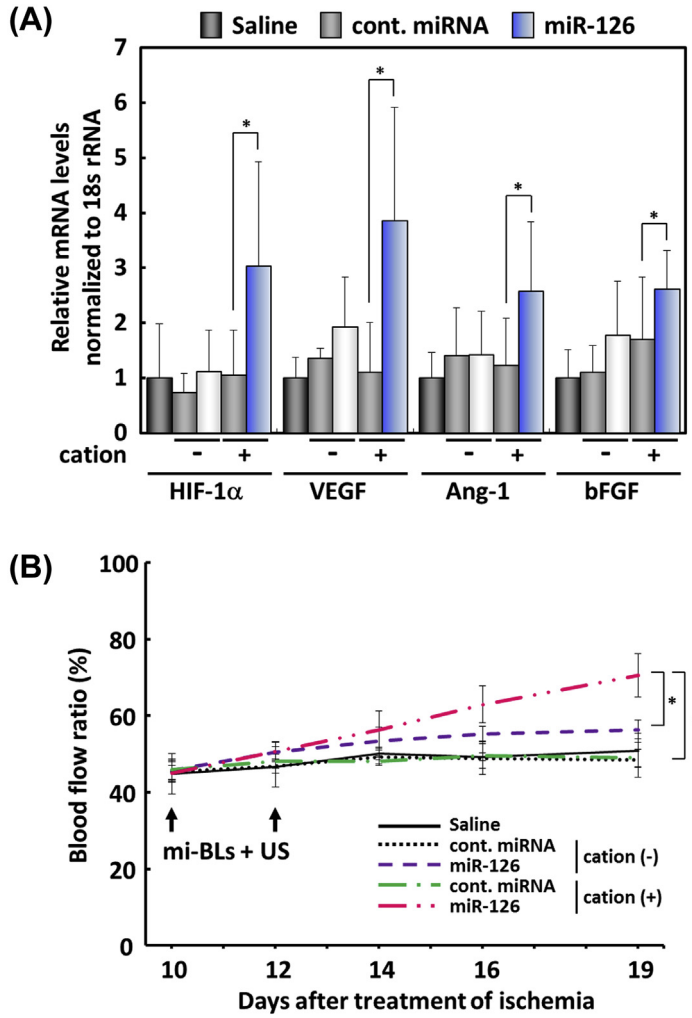

Figure 8 ***The therapeutic effects of miR-126 transfer by miRNA-loaded BLs (mi-BLs) and ultrasound (US) exposure on mice with hindlimb ischemia***. Ten days after femoral artery ligation, mice were treated with mi-BLs and US. The treatment was administered via tail vein injection twice daily every 2 days to mice with hindlimb ischemia. We injected a solution of miRNA (40 μg of miCont or miR-126) and BLs (200 μg). (A) The effect of miR-126 transfer by mi-BLs and US on mRNA expression for angiogenic genes. Seven days after the second transfection, RNA was isolated from the thigh muscle and analyzed using real-time PCR. (B) The effect of miR-126 transfer using mi-BLs and US on the recovery of blood flow. After the second transfection, blood flow was measured using a laser Doppler blood flow meter. All data are reported as the mean ± SD ($n = 4–6$). *Indicates $P < 0.05$ using a one-way analysis of variance with Tukey's post hoc test. *Reprinted from Endo-Takahashi et al. (2014) with permission.*

compatible with cellular internalization, endosomal escape, nuclear transfer, and transcription (Hama et al., 2006; Varga et al., 2005). Endosomal escape is one of the most important steps for improving intracellular trafficking of vectors. Indeed, the sensitivity to pH, temperature, or light have been used as approaches for delivering genes to the cytoplasm from endosomes.

Ultrasound has various biological effects, such as inducing calcium ion influx, generating reactive oxygen species, and activating cellular level signals (Juffermans, Dijkmans, Musters, Visser, & Kamp, 2006; Juffermans, Kamp, Dijkmans, Visser, & Musters, 2008; Takeuchi et al., 2008; Zhou, Shi, Cui, & Deng, 2008). We therefore built on our experience combining BLs with US for gene delivery and designed an enhanced endosomal escape system using BLs and US (Negishi, Omata, Iijima, Hamano, et al., 2010; Negishi, Omata, Iijima, Takabayashi, et al., 2010; Omata et al., 2011; Omata, Negishi, Hagiwara, et al., 2012; Omata, Negishi, Yamamura, et al., 2012).

Specific interactions between a ligand and receptor should provide a selective and efficient strategy for delivering genes to targeted tissues and cells. We developed AG73-labeled PEG liposomes encapsulating pDNA (AG73-PEG liposomes) to deliver genes to a tumor selectively (Negishi, Omata, Iijima, Hamano, et al., 2010). AG73 is peptide derived from laminin α1 chain and is a ligand for syndecans, a family of heparin sulfate-containing transmembrane proteins (Nomizu et al., 1995). Tumor cells highly express syncdecan-2 (Fears & Woods, 2006). We confirmed that AG73-PEG liposomes can target syndecan-2 overexpressing tumor cells. However, transfection with AG73-PEG lisposomes in the presence chloroquine, an endosomolytic reagent, resulted in increased gene expression compared to in the absence of chloroquine, suggesting that AG73-PEG liposomes may not efficiently deliver genes from endosomes to the cytoplasm. Therefore, we assessed the utility of BLs and US for enhancing the endosomal escape of gene delivery vectors and increasing transfection efficiency.

We prepared AG73-PEG liposomes encapsulating pcDNA3-Luc, an expression vector encoding the luciferase gene, and evaluated the effect of BLs and US on luciferase expression by AG73-PEG liposomes. Syndecan-2 overexpressing cells were incubated with AG73-PEG liposomes and then treated with BLs and US. Luciferase activity following treatment with BLs and US was about 60-fold higher than with AG73-PEG liposomes alone (Figure 9). We also confirmed that BLs and US did not increase the uptake of pDNA into cells. These results suggest that BLs and US can enhance the transfection efficiency of AG73-PEG liposomes without increasing the uptake of pDNA into cells, and that BLs combined with

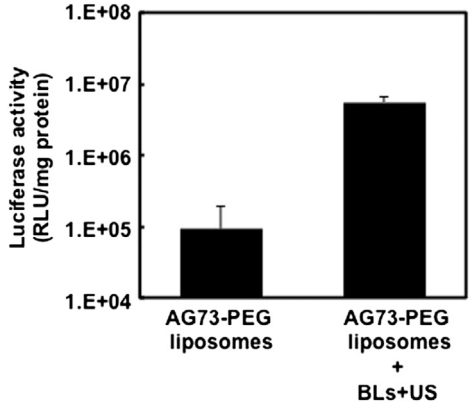

Figure 9 ***Effect of bubble liposomes (BLs) and ultrasound (US) exposure on gene transfection***. AG73-PEG liposomes containing pDNA were added to cells. After 4 h incubation, cells were washed and BLs were added. Immediately, US was exposed to cells. The cells were cultured for 20 h and then luciferase activities were measured. Data are shown as mean ± SD.

US outside cells can affect intracellular events. To investigate the mechanism of enhanced gene transfection with BLs and US, we evaluated the effects of BLs and US on intracellular trafficking of AG73-PEG liposomes encapsulating Cy3-labeled pDNA. Cells were incubated with AG73-PEG liposomes and Alexa Fluor 488-conjugated transferrin as an endosome marker, and then treated with BLs and US. The intracellular distribution of Cy3-labeled pDNA and Alexa Fluor 488-conjugated transferrin was observed by confocal microscopy. When cells were treated with AG73-PEG liposomes alone, the fluorescence of pDNA and transferrin was colocalized. In contrast, when cells were treated with AG73-PEG liposomes, BLs and US, the fluorescence of pDNA colocalized with transferrin was decreased. We also assessed the ratio of colocalization of pDNA and transferrin by measuring their pixels. Treatment with AG73-PEG liposomes, BLs and US decreased the ratio of colocalization of pDNA and transferrin compared with AG73-PEG liposomes alone. In addition, we confirmed that BLs and US reduced the ratio of colocalization of pDNA and LAMP-2, a lysosome marker. These results suggest that BLs and US can affect the intracellular trafficking of AG73-PEG liposomes and enhance endosomal escape of pDNA.

We evaluated the involvement of calcium ions (Ca^{2+}) to better understand the mechanism of enhanced endosomal escape by BLs and US. US induces Ca^{2+} influx by enhancing the permeability of the cell membrane, and

Ca^{2+} is involved in endosomal acidification and vesicle fusion (Fan, Kumon, Park, & Deng, 2010; Lelouvier & Puertollano, 2011; Mayorga et al., 1994). The involvement of adenosine triphosphate (ATP) in enhancing endosomal escape was also assessed. In the presence of ethylene glycol tetraacetic acid (EGTA) (added to chelate Ca^{2+}), luciferase activity was not increased by BLs and US compared with that in the absence of EGTA. We also evaluated the intracellular localization of Cy3-labeled pDNA and Alexa Fluor 488 transferrin by confocal microscopy. When cells were treated with AG73--PEG liposomes, BLs and US in the presence of EGTA, the coloclization of pDNA and transferrine fluorescence remained unchanged. Similar results were obtained when cells were treated with reagents (NaN_3, NaF, antimycin A) to deplete ATP. These results suggest that Ca^{2+} and ATP are necessary for enhancing endosomal escape by BLs and US.

Our study suggests that BLs outside cells, combined with US, can increase transfection efficiency by enhancing the endosomal escape of vectors (Figure 10). Thus, BLs and US may be a useful system in enhancing the endosomal escape of gene delivery vectors.

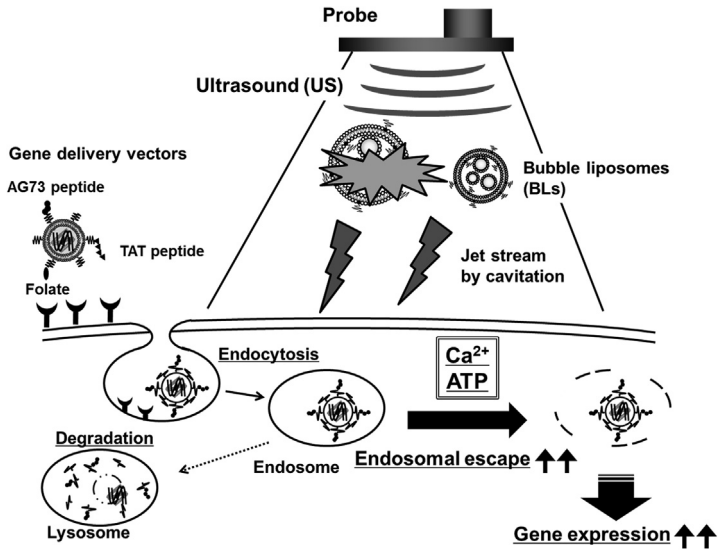

Figure 10 ***Enhanced endosomal escape of gene delivery vectors by bubble liposomes (BLs) and ultrasound (US).*** Some gene delivery vectors are internalized by the endocytic pathway and need to deliver genes to the cytoplasm from the endosome to achieve efficient gene transfection. By combining these vectors with BLs and US outside the cells, the endosomal escape of internalized vectors is enhanced and transfection efficiency is increased.

10. CONCLUSIONS

Ultrasound has long been used as a useful diagnostic tool. Therapeutic US was recently developed and is being used in clinical settings. The combination of therapeutic US and nano/microbubbles is an interesting and important system for establishing a novel and noninvasive gene delivery system. Genes are delivered more efficiently using this system compared with a conventional nonviral vector system such as the lipofection method, resulting in higher gene expression. This higher efficiency is due to the gene being delivered into the cytosol and bypassing the endocytosis pathway. Many *in vivo* studies have demonstrated US-mediated gene delivery with nano/microbubbles, and several gene therapy feasibility studies for various diseases have been reported. In addition, nano/microbubbles can deliver genes site-specifically by the control of US exposure site. Therefore, it is expected that this technology could be used clinically as a novel gene delivery system.

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CHAPTER THREE

Electroporation-Mediated Gene Delivery

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Contents

Abstract

Electroporation has been used extensively to transfer DNA to bacteria, yeast, and mammalian cells in culture for the past 30 years. Over this time, numerous advances have been made, from using fields to facilitate cell fusion, delivery of chemotherapeutic drugs to cells and tissues, and most importantly, gene and drug delivery in living tissues from rodents to man. Electroporation uses electrical fields to transiently destabilize the membrane allowing the entry of normally impermeable macromolecules into the cytoplasm. Surprisingly, at the appropriate field strengths, the application of these fields to tissues results in little, if any, damage or trauma. Indeed, electroporation has even been used successfully in human trials for gene delivery for the treatment of tumors and for vaccine development. Electroporation can lead to between 100 and 1000-fold increases in gene delivery and expression and can also increase both the distribution of cells taking up and expressing the DNA as well as the absolute amount of gene product per cell (likely due to increased delivery of plasmids into each cell). Effective electroporation depends on electric field parameters, electrode design, the tissues and cells being targeted, and the plasmids that are being transferred themselves. Most importantly, there is no single combination of these variables that leads to greatest

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efficacy in every situation; optimization is required in every new setting. Electroporation-mediated *in vivo* gene delivery has proven highly effective in vaccine production, transgene expression, enzyme replacement, and control of a variety of cancers. Almost any tissue can be targeted with electroporation, including muscle, skin, heart, liver, lung, and vasculature. This chapter will provide an overview of the theory of electroporation for the delivery of DNA both in individual cells and in tissues and its application for *in vivo* gene delivery in a number of animal models.

1. INTRODUCTION

One of the great limitations of nonviral gene delivery has been perceived to be the relative lack of high-level gene transfer *in vivo*. Many nonviral techniques, while much safer in terms of inflammatory and immune responses to the vectors themselves, have generally resulted in much lower levels of gene delivery and expression when compared to their viral counterparts. However, one nonviral approach has proven both safe and highly effective in high-level gene transfer: electroporation. Electroporation uses electric field to facilitate the delivery of nucleic acids and other impermeable molecules to cells. Perhaps the most transformative of these molecules, in terms of biology and medicine, is DNA. Neumann and colleagues first demonstrated that moderate electric fields could be used to transfect mammalian cells in culture with plasmids in 1982 and formalized a mathematical and electrical theory for how the process occurred (Neumann, Schaefer-Ridder, Wang, & Hofschneider, 1982). In the past 30 years, numerous advances have been made in the field, from using fields to facilitate cell fusion, transformation in bacteria, delivery of chemotherapeutic drugs into cells and tissues, and most importantly, gene and drug delivery into living tissues from rodents to man (Ausubel et al., 1999; Heller & Heller, 2006; Potter, Weir, & Leder, 1984). Electroporation-mediated *in vivo* gene delivery has proved highly effective in vaccine production, transgene expression, enzyme replacement, and control of a variety of cancers. When a square-wave electric field at the appropriate strength is applied to any number of tissues such as skin, liver, muscle, and a variety of tumors, following direct injection of the plasmid, the levels of gene expression jump between 20- and 1000-fold (Aihara & Miyazaki, 1998; Cemazar et al., 2009; Heller et al., 2000; Lin et al., 2012; Mathiesen, 1999; Mir et al., 1999; Wells, Li, Sen, Jahreis, & Hui, 2000). Most exciting is the fact that a number of Phase I and Phase II clinical trials are either underway or have been completed, clearly demonstrating the efficacy of this

approach. This chapter will provide an overview of the theory of electroporation for the delivery of DNA both into individual cells and in tissues and its application for *in vivo* gene delivery in a number of animal models.

2. THEORY OF MEMBRANE ELECTROPORATION

The hydrophobic lipid bilayer of the plasma membrane can be thought of as a simple capacitor that stores a charge and acts as a dielectric between the highly charged conductive environments of the extracellular medium and the cytoplasm. When cells are exposed to an electric field, the membrane can build up a charge in the form of a transmembrane potential (Figure 1(A)). The electric field causes the dipoles of molecules from proteins to carbohydrates to orient themselves with respect to the field. They then distribute within and around the cell such that the side of the cell facing the cathode is "depolarized," and the other facing the anode is "hyperpolarized" due to the differential accumulation of charge on either side of the plasma membrane (Hibino, Itoh, & Kinosita, 1993). Once this electric field-induced transmembrane potential exceeds the dielectric

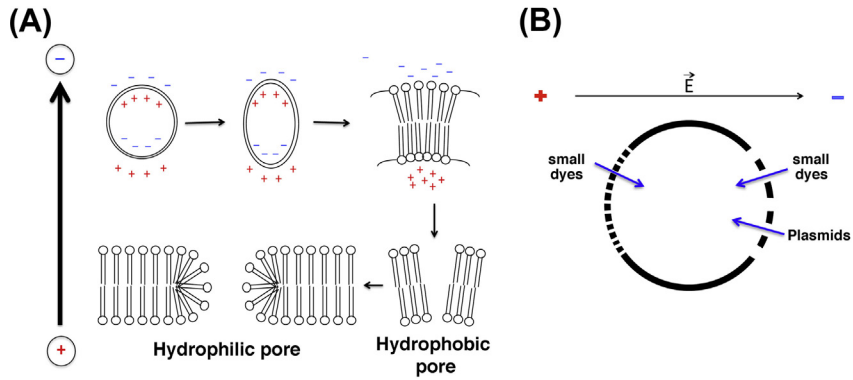

Figure 1 ***Model for electropermeabilization and electroporation***. (A) The presence of an applied electric field induces movement of and redistribution of macromolecular dipoles within and outside the cell, resulting in accumulation of charges across the membrane. Once the transmembrane potential exceeds the dielectric strength of the membrane, transient permeation events occur, generating small hydrophilic pores that stabilize and coalesce into larger pores to allow movement of large molecules such as DNA. (B) The permeabilization of the cell induced by the electric field occurs differentially with respect to pore size: a number of small pores are formed at both poles of the cell, but larger pores capable of allowing DNA entry only form at the anode-facing pole. *Modified from Somiari et al. (2000) and Escoffre, Rols, et al. (2011).*

strength of the membrane (typically around 500 mV), the membrane undergoes a permeation event resulting in the formation of hydrophobic pores that allow water movement and limited ion flow. With increasing time in the field, the destabilized membrane and the hydrophobic pores stabilize to form larger pores that allow the entry and exit of larger impermeable molecules in and out of the cell. Indeed, a study of human embryonic stem cells shows that they required short pulse times of 0.05 ms for loading of propidium iodide and other small molecules, while pulse times of 0.5 ms or more were required for DNA transfection using the same field strengths (Mohr, de Pablo, & Palecek, 2006). Once the field is removed, the pores destabilize and close over time (minute scale), allowing the membrane to reseal.

Computer simulations using recent advances in molecular dynamics approaches to describe membrane behavior under the influence of an applied electric field have suggested that once the membrane reaches the critical point, water molecules begin to gain access across the membrane and an inverted hydrophilic pore can be formed (Figure 2) (Delemotte & Tarek, 2012; Ho, Casciola, Levine, & Vernier, 2013; Ho, Levine, & Vernier, 2013; Kramar et al., 2012; Polak et al., 2013; Tokman et al., 2013). With continued application in the field, this metastable hydrophilic pore can transition to a larger, more stable pore that can allow the transport of larger charged macromolecules. While considerable disagreements continue in the field as to whether pores actually form (they have not been

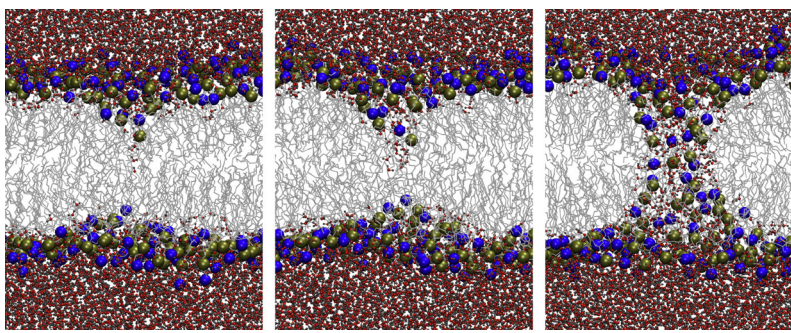

Figure 2 ***Molecular dynamics solution of the formation of hydrophilic water channels in a membrane in response to an applied electric field.*** Snapshots of the time evolution of water–lipid–water configurations under an external electric field of 500 MV/m. Panels (left to right) are 5.8, 6.7, and 7.3 ns from the start of the simulation with both water molecules (oxygen—red, hydrogen—gray) and lipid molecules (phosphorus—yellow, nitrogen—blue, lipid tail groups—silver) displayed. *Reprinted with permission from Tokman et al. (2013).* (For interpretation of the references to color in this figure legend see the color plate.)

directly visualized experimentally), there is overwhelming agreement that membrane permeation, resulting in entry of macromolecules, does occur.

The mathematical theory first proposed by Neumann assumes a completely spherical model cell that becomes slightly elongated with the applied electric field, leading to permeation events at the poles (Figure 1) (Neumann et al., 1982). The model proposes that the permeation events occur perpendicular to the field at either apex of the slightly elliptical cells. Experimentation using small fluorescent dyes and either membrane vesicles or isolated cells has shown that this appears to be the case. The entry of small molecules and dyes does not occur uniformly around the cell, but rather at the two opposing sides of the cell within the field (Figure 1(B)) (Golzio, Teissie, & Rols, 2002; Hibino et al., 1993; Tekle, Astumian, & Chock, 1994). As cells move away from the theoretical spheroid shape to more realistic ones with various protrusions and bulges, the distribution of the field and its effects on the transmembrane potential becomes more heterogeneous (Valic et al., 2003). This results in a greater permeabilization at the ends of protrusions with great degrees of curvatures (*i.e.*, at the end of a skeletal myotube or neuron) than along the remainder of the cell body. Additionally, the size of the cell can have a major impact on its ability to be electroporated. As the diameter of the cell increases, the external field needed for effective electroporation decreases. This may also explain why certain cells within tissues can be electroporated using reduced field strengths; if adjacent cells are electrically connected via gap junctions, they may act as a single functionally large cell. Thus, muscle fibers which can be treated as one large "cell" with a number of distinct, but not isolated, myotubes, typically require much lower field strengths for effective electroporation-mediated gene delivery as compared to a variety of other cell and tissue types (Mir et al., 1999).

3. MECHANISM(S) OF ELECTROPORATION-MEDIATED GENE DELIVERY

For electroporation-mediated gene delivery to occur, two things need to be simultaneously present: nucleic acid and the electric field. DNA and other nucleic acids must be present in the tissue or cell suspension before or during the application of electric pulses. In contrast, small molecules and dyes such as propidium iodide or trypan blue can enter cells when added during, or shortly after, application of the electric field and still gain access to cells. When trypan blue was added to adherent cells that had been

electroporated at various times, 50% of the cells excluded the dye 6 min after pulsing, however, all cells excluded the dye by 30 min (Gabriel & Teissie, 1995; Rols & Teissie, 1990). This is likely due to the fact that DNA is much larger than any small reporter dye and thus requires larger functional pores for its entry (Figure 1(B)). As the membrane destabilizes and the permeation event occurs, small pores open that then coalesce into larger ones. Increasing the pulse duration or number of pulses can lead to greater functional pore sizes, but the majority of these pores likely close faster than the larger number of small pores, in a simple exponential fashion. Thus, if DNA is not present at the time of the pulsing, all the pores capable of allowing its transport will be closed by the time of DNA addition.

Experiments with fluorescent dyes have shown that electric field-induced permeation of the membrane occurs at the poles of the cell perpendicular to the applied field. Visualization of the accumulated dye appears rather uniform at these poles, suggesting transport across the membrane at a number of locations within these areas (Escoffre et al., 2009). While DNA also enters the cells at these same poles with respect to the applied electric field, the distribution of DNA at the membrane is not uniform like propidium iodide, but rather is punctate (Escoffre, Rols, & Dean, 2011; Faurie et al., 2010; Golzio et al., 2002). These DNA "spots" aggregate at the pole facing the anode and are between 0.1 and 0.5 μm in size. The fact that DNA accumulates on the anode-facing pole suggests that the electric field causes electrophoretic movement of DNA to this face of the cells (Figure 1(B)). While the number of these DNA spots at the membrane does not increase, they do appear to grow in size as the field is applied, but once the field is removed, the growth halts (Escoffre, Portet, et al., 2011; Golzio et al., 2002). These spots remained visible at the membrane for at least 10 min after removal of the electric field, but by this time the DNA was inaccessible to the extracellular environment. When a DNA-staining dye, TOTO, was added to electroporated cells within several seconds of removal of the field, the DNA at the anode pole was able to bind the dye, suggesting that it is bound to the surface of the cell and not yet internalized. By 30 min after electroporation, the DNA spots had migrated to areas within the cell, and the majority of the fluorescent DNA still appeared to remain in these focused areas as opposed to being diffused throughout the cell. However, if the dye was added 10 min after cessation of the pulses, no staining of DNA by TOTO was detected (Golzio et al., 2002).

The mechanism by which DNA actually enters the cell remains unknown. A number of theoretical studies suggest that DNA gains direct entry

into the cytosol, and experiments with planar lipid bilayers and giant unilamellar vesicles have also shown that electroporation can cause direct entry of DNA across the membrane (Hristova, Tsoneva, & Neumann, 1997; Portet et al., 2012, 2009; Riske & Dimova, 2005). However, studies with intact cells are not as clear. The fact that DNA accumulates, concomitant with electroporation, in spots at the membrane that cannot be removed upon reversal of the polarity of the electric field has led to the conclusion that DNA must be trapped within lipid vesicles at the membrane (Golzio et al., 2002). Several groups have interpreted these findings to suggest that DNA is endocytosed or at least encased within electroporation-induced membrane vesicles. In support of this, studies have shown that, although depletion of cholesterol from cells does not alter the number of DNA aggregates/spots that appear at the hyperpolarized membrane upon electroporation, it can decrease transfection efficiency by three- to sixfold in the same cells. Since cholesterol depletion inhibits both clathrin- and caveolae/lipid raft-mediated endocytosis, these results have been interpreted to imply that inhibition of these two modes of endocytosis reduces the internalization of DNA. However, it should be pointed out that no study has directly followed labeled DNA across the membrane in these types of cholesterol depletion studies; therefore, reliance on downstream expression as indicators of distinct pathways of uptake is not definitive. Moreover, if DNA enters the cell via electroporation-stimulated endocytosis, it is unclear whether this population of DNA is actually responsible for productive transfection. In other words, any DNA that enters the cell via electroporation-induced endocytosis could represent a dead end for transfection, whereas DNA entering the cytoplasm by direct translocation could drive all expression seen. Similar dead-end cell entry pathways have been described and debated for a number of viruses over the years (Sieczkarski & Whittaker, 2005).

Once translocated into the cytosol, DNA must make its way to the nucleus in order to be expressed (Figure 3). Since as early as the 1940s, the cell cytoplasm has been described as resembling a reversible gel–sol system (Chambers, 1940). It is a complex system composed of multiple cytoskeletal elements, including actin microfilaments, microtubules, and intermediate filaments. These elements are organized into a complex, crowded latticework that is constantly remodeling in response to a variety of internal and external stimuli. A number of studies have demonstrated that passive diffusion of either double-stranded DNA fragments greater than 1000–2000 bp or macromolecule-sized solutes in HeLa cells and fibroblasts is essentially nonexistent (Lukacs et al., 2000; Seksek, Biwersi, & Verkman,

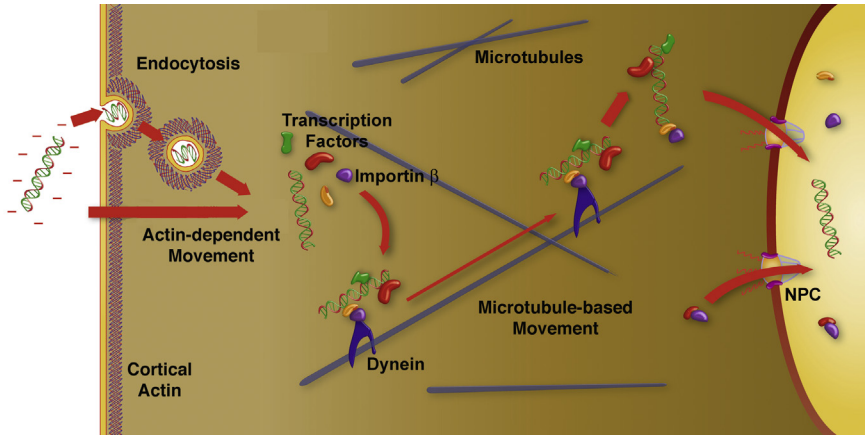

Figure 3 ***Postelectroporation trafficking of plasmids to the nucleus during gene transfer.*** Following electropermeabilization of the membrane, plasmids may enter the cell by either endocytosis and/or direct entry into the cytosol (Rosazza et al., 2013; Rosazza et al., 2011). The initial trafficking events near the cell surface appear to involve actin and actin-based movement. Once through the cortical actin layer and free in the cytoplasm, plasmids are rapidly complexed by a number of DNA-binding proteins present in the cytoplasm which in turn bind to other proteins to form large protein–DNA complexes (Badding et al., 2013). Transcription factors bound to DNA interact with importin β and other proteins that in turn link the complex to dynein for movement along microtubules to the nucleus where it falls apart at the nuclear periphery (Badding et al., 2012). Nuclear entry is then mediated by importin β in a sequence- and importin-dependent manner. (For interpretation of the references to color in this figure see the color plate.)

1997). Thus, in order to traffic to the nucleus, directed movement must be employed. Indeed, others and we have shown that plasmids utilize the microtubule network and its associated proteins to move to the nucleus (Badding & Dean, 2013; Badding, Lapek, Friedman, & Dean, 2013, Badding, Vaughan, & Dean, 2012; Geiger, Taylor, Glucksberg, & Dean, 2006; Rosazza et al., 2013; Vaughan & Dean, 2006; Vaughan et al., 2008). Within 15 min of electroporation in adherent cells, transfected plasmids have been shown to physically interact with transcription factors, including cAMP response element-binding protein (CREB); this binding is necessary for maximal movement along microtubules (Badding et al., 2012). Since CREB is present only in the nucleus and cytoplasm, its interaction with DNA suggests that at least a fraction of the electroporated DNA has entered the cytosol by this time. Once the DNA has been released into the cytoplasm (either upon escape from any endosomes or after direct entry across the plasma membrane, depending on which mechanism

is predominant and productive for transfection), it binds to a number of sequence-specific and nonspecific DNA-binding proteins as well as a number of other proteins to form a large protein–DNA complex (Badding et al., 2013; Miller, Munkonge, Alton, & Dean, 2009; Munkonge et al., 2009). These additional proteins include a number of microtubule accessory proteins and the motors dynein and kinesin, as well as tubulin itself. Real-time particle tracking of quantum dot-labeled plasmids has shown that DNA moves along microtubules with kinetics and dynamics that are in line with those seen for microtubule-based movement of organelles, virus particles, and proteins (Badding & Dean, 2013; Badding et al., 2013, Badding et al., 2012; Rosazza et al., 2013). Further, inhibition of dynein using antibodies or destabilization of microtubules using nocodazole abolishes the ability of DNA to move toward the nucleus (Rosazza et al., 2013; Vaughan & Dean, 2006).

Several recent studies have also implicated a role for actin filaments in the electroporation-mediated cell entry and/or cytoplasmic trafficking of plasmids in addition to that of the microtubule network (Rosazza et al., 2013, Rosazza, Escoffre, Zumbusch, & Rols, 2011). In experiments in which either green fluorescent protein (GFP)-actin or phalloidin-rhodamine-labeled actin was visualized along with DNA immediately or at various times after electroporation, both DNA and actin colocalized to the same spots/aggregates at the permeabilized membrane facing the anode (Rosazza et al., 2011). When latrunculin B was used to destabilize the actin network, the same number of individual DNA aggregates was still seen at the same membrane locations with respect to the electric field, but only about half as much DNA was present at the spots. In a follow-up study, actin dynamics were perturbed with either latrunculin B or jasplakinolide, and the intracellular movement of electroporated plasmids was followed by single-molecule particle tracking (Rosazza et al., 2013). In this study, either depolymerization or stabilization of the actin network caused slight decreases in the percent of particles showing active transport or the total distance traveled by the particles, respectively. Proteomic studies from our laboratory have found that several actin-based motors (myosin 1B, 1C, and 9) are found in the protein–DNA complexes at early times after electroporation (15 min) along with a number of different microtubule-based motors (Badding et al., 2013), supporting a possible role for actin-based movement of DNA particles, at least at times between entry of DNA into the cytosol and binding to the microtubules.

Following the trafficking of plasmids to the interior of the cell, they must enter the nucleus for productive gene expression to take place. As for

microtubule-based movement, it is the proteins that bind to the plasmids that mediate entry into the nucleus in nondividing cells following electroporation or any transfection method. Others and we have shown that when plasmids carry certain DNA sequences termed DNA nuclear targeting sequences (DTS), they form complexes with specific nuclear localization signal-containing proteins that in turn bind to importins for entry of the complex through the nuclear pore complex into the nucleus (Cramer et al., 2012; Dean, 1997; Dean, Dean, Muller, & Smith, 1999; Degiulio, Kaufman, & Dean, 2010; Langle-Rouault et al., 1998; Mesika, Grigoreva, Zohar, & Reich, 2001; Sacramento, Moraes, Denapolis, & Han, 2010; Vacik, Dean, Zimmer, & Dean, 1999). The most common proteins that bind to these sequences are transcription factors which are translated and many times retained in the cytoplasm to regulate their function (Wente & Rout, 2010). If a plasmid containing a DTS is present in the cytoplasm, these cytoplasmic transcription factors can bind to this site before nuclear import to facilitate translocation into the nucleus (Badding et al., 2013, 2012; Miller et al., 2009; Miller & Dean, 2008). Alternatively, any plasmid, regardless of sequence, has the ability to enter into the nucleus during mitosis, once it reaches the area of the nuclear periphery. Either way, these trafficking events through the cytoplasm and into the nucleus are not unique to electroporation, but are seen in all other nonviral methods for gene delivery.

4. PULSE PARAMETERS

Two types of wave forms are used in electroporation of cultured cells: square wave and exponential decay. As for any transfection method, optimization is necessary to determine which works best for desired cell types. Square-wave electroporators deliver pulses to cells with a set voltage for a defined amount of time. These pulses typically last for between 100 μs and 100 ms, and fields are usually between 100 and 1300 v/cm (100–500 V in a 0.4 cm cuvette). Exponential decay electroporators deliver a peak of energy that dissipates exponentially, giving a time constant (τ), a function of the resistance of the sample and the capacitance set on the instrument and which corresponds to the time necessary for the charge to decrease to about 37% of the initial voltage. While both wave forms work well for bacteria, yeast, and cultured mammalian and plant cells in suspension and in *in vivo* applications in living tissues, square waves have been consistently shown to be safer, induce less damage, and yield better gene transfer and expression.

It has been shown that the duration of the square-wave pulse, the number of pulses, the magnitude of the field, and the frequency with which the pulses are applied, can all influence electroporation-mediated gene delivery (Gehl & Mir, 1999; Gehl et al., 1999; Zaharoff, Henshaw, Mossop, & Yuan, 2008). Studies have shown that several different combinations of pulse lengths and field strengths can be used for electroporation in skeletal muscle and tumors (Lucas, Heller, Coppola, & Heller, 2002; Satkauskas et al., 2002). In several seminal papers using electroporation, high field strengths (1000–2000 V/cm) and multiple short pulses (<100 μs) were used (Heller et al., 1996; Titomirov, Sukharev, & Kistanova, 1991), but lower field strengths (200 V/cm) with multiple longer pulses (10–20 ms) can work in a number of tissues as well (Aihara & Miyazaki, 1998; Mathiesen, 1999; Mir et al., 1999). A more recent report from Mir has suggested that a combination of the two is even more effective in gene transfer (Satkauskas et al., 2002). Regardless of the specifics of low field/long pulses, high field/short pulses, or combinations, empirical optimization by the investigator must be carried out for every tissue and every DNA that will be used. Thus, there is no single "optimized" set of pulse conditions.

Since cell shape, size, and composition can all play a role in how cells sense and respond to the electric field, it is possible to use the field to limit permeabilization and electroporation to specific cell types within a tissue. For example, since larger cells are permeabilized at lower field strengths than smaller cells, it is possible to electroporate specific cells within a tissue if they are sufficiently different in size than other cells within the tissue (Valic et al., 2003). In one study, this fact was exploited to specifically target lipid-laden adipocytes which are much larger (>fivefold) than other cells in adipose tissue for gene transfer. When a series of seven, 20 ms pulses at 500 V/cm were administered to suprascapular white adipose tissue in mice, 99% of cells expressing the delivered transgene were adipocytes, which made up only 16% of the cells within the tissue (Granneman, Li, Lu, & Tilak, 2004).

One major misconception of electroporation, or any gene delivery method for that matter, is that the ultimate goal is to achieve as high a level of gene expression as possible. Indeed, researchers routinely optimize delivery parameters using either luciferase-expressing constructs to measure absolute levels of gene expression or GFP-expressing constructs to measure the fraction of cells expressing their DNA. While maximization of gene transfer and expression is understandable in light of the perception that nonviral gene delivery is "less efficient" than viral gene delivery and that there is a need to

reach these viral levels of gene transfer and expression in many cases, or perhaps even all cases, obtaining the maximal amount of a transgene may not be the best approach for therapeutic needs. For example, in studies from our laboratory several years ago, we delivered plasmids expressing subunits of the Na^+/K^+-ATPase to the lungs of mice and rats with the goal of achieving the same levels of transgene expression as seen using adenoviral vectors to transfer the same genes (Factor et al., 1998; Machado-Aranda et al., 2005). Even under "optimized" conditions of electroporation-mediated gene delivery, an adenoviral vector still gave roughly fivefold more expression of the gene product as determined by western blot. However, when we measured the physiological response mediated by the transferred gene product, both adenoviral and electroporation-mediated transfer gave equivalent activities, which is the ultimate goal. Thus, as the influential twentieth-century modernist architect Ludwig Mies van der Rohe said, often "less is more."

5. ELECTRODES

Any number of electrode types can be used in applying the electric field—the choice is dependent on the tissue and the goals for expression that are desired. The first types of electrodes for cultured cells were parallel plates inserted into a solution of suspended cells. This was followed up by incorporating the plates into the walls of cuvettes, leading to the electroporation cuvettes that are widely used today. Since animals or parts of them could not be jammed into these cuvettes, other electrode configurations were developed (Figure 4). The first two general designs were based on surface-applied plate electrodes (configured as tweezers or calipers) and penetrating needles for application into the tissue to be electroporated (Gilbert, Jaroszeski, & Heller, 1997). Needle electrodes have come in multiple flavors, from two needles that can be inserted to varying depth to multiple needle arrays containing six or more needles that fire in defined patterns to deliver the field with changing polarity to maximize cell permeabilization, DNA electrophoresis, and gene delivery (Jaroszeski, Gilbert, & Heller, 1997). The needles can be insulated along their shaft with only their tips being able to generate the field. These types of needles are excellent in delivering fields at precise depths within a tissue and act as point charges. The maximal field delivered from these needles is at the tip and falls off as the distance from the tip increases (Sel, Mazeres, Teissie, & Miklavcic, 2003).

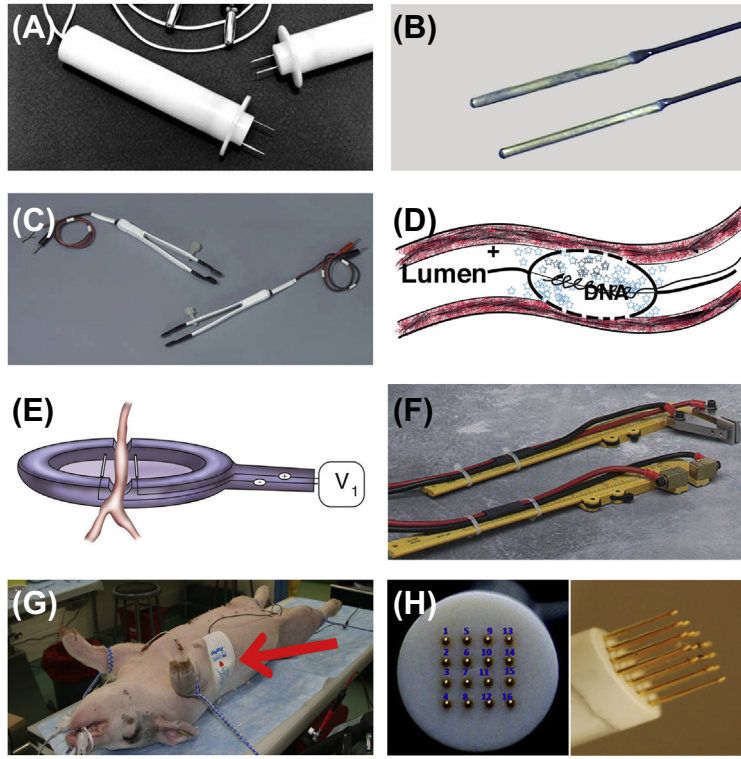

Figure 4 ***Examples of electrodes for electroporation.*** (A) Penetrating, two-needle arrays. (B) Nonpenetrating parallel needles (Genetrode electrodes, Genetronics, San Diego, CA, USA). (C) Plate electrodes (Tweezertrodes, BTX, Hollister, MA). (D) Cartoon of a balloon catheter-based electrode for delivery of DNA and electroporation. (E) Spoon electrode for vascular electroporation. (F) Caliper-mounted plate electrodes. (G) Conformable defibrillator pads for electroporation (arrow). (H) Multielectrode array (R. Heller, personal communication). (For interpretation of the references to color in this figure see the color plate.)

Such insulated needles can be inserted through the skin into a specific muscle to deliver the electric field only to the muscle and not to all cells and tissues along the shaft. Alternatively, uninsulated needles are very well suited for delivering the field over a wider area (all along the length of the needle at all depths). Since electroporation only occurs where the electric field and DNA overlap, some groups and companies have incorporated needles for DNA delivery into the needle arrays.

Like penetrating needle electrodes, a number of plate electrodes have been developed from simple plates that are placed on the skin to those constrained by tweezers or calipers. The advantage to this type of electrode is that

the field applied between the plates is larger and more uniform (Gehl et al., 1999). Again, as in needle arrays, a four-plate electrode has been developed for skin electroporation that is highly effective and can be used to rotate the applied field leading to greater gene delivery (Heller et al., 2007). While these plate electrodes are rather small using electrode surface areas of less than 1 cm^2, larger plate electrodes can also be used safely for electroporation of larger organs or animals. For example, we have used defibrillation paddles and defibrillation electrode pads (10 × 15 cm) to deliver fields across the chests of 40-kg pigs for efficient gene delivery into the lungs (Dean, Barravecchia, Danziger, & Lin, 2011; Emr et al., in press).

Other designs include electrodes based on electroporation cuvettes. Since the vascular wall of vessels, with the exception of very large arteries such as the aorta cannot be injected with DNA, we designed an electrode that resembles a spoon in which the vessel segment to be electroporated can be placed and bathed in a DNA solution (Martin, Young, Benoit, & Dean, 2000; Young & Dean, 2002). Wires on either side of the vessel act as electrodes to apply the field. Various sizes of this electrode have been used to transfect small mesenteric neurovascular bundles in mice (less than 0.5 mm diameter) and rats (~1 mm diameter), to the rat carotid (3 mm), and even segments of mouse and rat small intestine (up to 0.8 cm diameter).

Another innovative electrode design was developed by Heller for use in the skin (Donate, Coppola, Cruz, & Heller, 2011; Ferraro et al., 2011; Guo et al., 2011; Heller et al., 2010). One negative side effect of electroporation is that the delivery of electric pulses into skin or muscle is accompanied by minor pain that is proportional to the field strength. Thus, if the field strength (and/or pulse length) can be reduced, less pain will be experienced, making the approach more clinically acceptable. The approach taken by the Heller group was to reduce the distance between electrodes so that lower voltages can be applied to the skin, while increasing the number of individual electrode pairs to increase the size of the area to be electroporated. The noninvasive multielectrode array (MEA) has 16 electrodes placed 2 mm apart in a 4 × 4 array (Heller et al., 2010). Thus, to achieve a field of 250 V/cm, only 50 V needs to be applied between electrode pairs as opposed to 200 V for the 8 mm total gap. Pulses are administered in a sequence that utilizes four electrodes at a time forming 2 × 2 mm squares (nine total squares). Pulses are applied in pairs, in two directions, perpendicular to each other (18 pulses) for four rounds of pulsing (72 total pulses). Using this approach, high levels of gene expression can be obtained in the skin that are equal to that seen using

plate electrodes with the benefit of using low applied energy for reduced sensation (Heller et al., 2010).

6. TISSUES THAT CAN BE DIRECTLY INJECTED: LIVER AND SKELETAL MUSCLE

Unlike transfection of cultured cells, it has been demonstrated that direct injection of DNA without any carrier or other physical method can result in gene transfer and expression of transgenes in a number of tissues. In 1990, Harold Varmus' group showed that plasmids directly injected into the livers of ground squirrels showed gene expression within 8 weeks in the absence of any carrier (Seeger, Ganem, & Varmus, 1984). Similarly, in the same year, Jon Wolff showed that plasmids can be directly injected into mouse skeletal muscle and drive relatively high-level gene expression within 8 h that lasted for at least 2 months (Wolff et al., 1990). These studies went on to form the basis for all studies in DNA vaccines. Other organs that have shown expression following injection of naked DNA alone include the lung (Pringle et al., 2007), kidney (Barry et al., 1999; Wang, Chao, & Chao, 1995), heart (Li, Welikson, Vikstrom, & Leinwand, 1997; Wang et al., 1995), and tumors (Yang & Huang, 1996), among others. In all cases, the levels of gene transfer are low compared to methods of viral delivery or delivery of the plasmids complexed with lipids, calcium phosphate, or polymers. In a seminal paper, Richard Heller and colleagues showed in 1996 that direct injection of plasmid DNA into the rat liver and immediate application of six square-wave electric pulses (1000 V/cm, 99 μs each, 1 Hz) could increase gene expression by a factor of 10 compared to DNA injection alone (Heller et al., 1996). Field strengths less than this gave no increase in gene expression over DNA alone, and when a field strength of 2000 V/cm was used, necrosis of liver tissue was observed. Expression was detected within 48 h, persisted for at least 21 days, and was almost entirely localized to hepatocytes. Indeed, between 30% and 35% of hepatocytes in the electroporated liver were found to be positive in transgene expression. Taken together, these experiments demonstrated the utility of this simple procedure for enhanced gene delivery and were the basis for the first "optimized" parameters for electroporation: six to eight short pulses (100 μs) at high fields (1000–1300 V/cm).

In a series of papers published in the late 1990s, multiple groups showed that the application of electric pulses caused up to a 1000-fold increase in the levels of gene expression compared to DNA injection alone in mouse

skeletal muscle (Aihara & Miyazaki, 1998; Mathiesen, 1999; Mir et al., 1999). In all cases, DNA was purified and suspended in physiological saline (0.9% NaCl) and injected into mouse tibialis and soleus muscles. In most cases, between 30 and 60 μl of DNA was injected into the muscles that are about twice that in volume. It has been demonstrated that when small volumes are used, the distribution of gene expression is more focused around the site of injection and not dispersed evenly throughout the tissue. While not a major issue in small animals, as muscle electroporation is applied to larger species, this becomes an important variable. Immediately following DNA injection, a series of electric pulses were applied, in all cases using penetrating two-needle electrodes placed into the muscle. These early studies characterized the pulse parameters and outcomes to show that DNA expression was dose dependent with up to 20 ng of luciferase transgene being expressed per muscle by 2 days (Mir et al., 1999). Both the distribution of cells taking up and expressing DNA increases upon electroporation as does the absolute amount of gene product per cell (this is likely due to increased delivery of plasmids into each cell). In a different study, secreted IL-5 was expressed and similar doses of plasmid yielded 20–30 ng of IL-5 per ml of serum (since the blood volume of a mouse is approximately 2 ml, these expression levels are similar) (Aihara & Miyazaki, 1998). Delivery and expression were also dependent on pulse length with high-level expression seen between 10 and 50 ms per pulse, when using fields of 200 V/cm. As in liver, expression was field dependent with a threshold seen around 100 V/cm; below this, expression was similar to that without any applied field. Expression increased with fields between 100 and 200 V/cm and then dropped off at fields above 200 V/cm (Aihara & Miyazaki, 1998; Mir et al., 1999). Pulse number also influenced gene delivery. Even with one pulse (200 V/cm, 20 ms), expression was twofold higher than in non-electroporated muscles. Expression maximized with between six and eight pulses (about 10-fold higher than DNA alone). Thus, with these studies, the second "optimized" set of parameters was established using a series of eight pulses at around 200 V/cm with pulses at durations of around 20 ms.

Based on these studies with two tissues, two types of pulsing parameters were established for *in vivo* electroporation. In the seminal papers using electroporation, high field strengths (1000–1300 V/cm) and multiple short pulses (<100 μs) were used, while later papers shifted to lower field strengths (200 V/cm) with multiple longer pulses (10–20 ms). It should be stressed that neither type is better than the other for all tissues or DNAs to be expressed. Indeed, while early reports from Heller using the liver

demonstrated that the short-pulse, high-field combination worked very well, several later reports from different groups showed that longer duration, lower field pulses worked equally as well in liver (Jaichandran et al., 2006; Suzuki, Shin, Fujikura, Matsuzaki, & Takata, 1998) (it should be pointed out that this group used surface plate electrodes, see below). Reports from Mir and colleagues have suggested that a combination of the two is even more effective for gene transfer (Satkauskas et al., 2002). Since the function of the electric pulses has two components—to permeabilize the membrane and to electrophorese DNA, it was reasoned that a combination of one or more short high-voltage pulses (100 μs, 1000 V/cm) to permeabilize the membrane, followed by long (100 ms) low-voltage pulses for electrophoresis may be beneficial (Andre et al., 2008). While individual researchers have championed these various approaches and conditions, it is imperative to realize that optimization is crucial for any given tissue, electrode, plasmid, and expression goal for success.

7. ELECTROPORATION OF SKELETAL MUSCLE: DNA VACCINES AND USE AS BIOREACTORS

The ease and reproducibility of electroporation-mediated gene transfer to skeletal muscle have made it the most highly used target organ for this approach. Early excitement over the use of muscles to produce antigen for vaccine development following naked DNA delivery alone was amplified even more when electroporation could be incorporated as well. It was found that DNA injection alone into mouse skeletal muscle resulted in highly variable gene expression and subsequent humoral and cellular immune responses (Capone et al., 2006; Widera et al., 2000). These findings were recapitulated in higher animals including pigs and nonhuman primates (Babiuk et al., 2002; Zhao et al., 2006). One great advantage of electroporation is its greater reproducibility of gene delivery and expression, reducing variability between animals (Bettan et al., 2000). In the last 15 years, the use of electroporation-mediated DNA vaccination has exploded and moved to clinical trials and approved for veterinary use. For more detailed examples, several recent reviews are suggested (Gothelf & Gehl, 2012; Liu, 2011).

Following DNA injection and electroporation, plasmids are expressed throughout the muscle, resulting in transgene protein production in myocytes, resident dendritic cells, and monocytes that reside within the tissue. When dendritic cells are transfected directly, the produced transgene can be processed by the host cell machinery and presented with MHC class I

or II molecules on the antigen-presenting cell, causing the cells to prime naïve T cells in the draining lymph nodes. When the transgene products form complexes with MHC class I molecules and prime naïve CD8+ T cells, cellular CTL responses are generated. Alternatively, the resident dendritic cells can engulf apoptotic or necrotic transfected myocytes or capture antigen secreted from transfected cells, resulting in the presentation of transgene products with MHC class II molecules, and priming of the immune system.

Although electroporation is touted as being extremely safe at the appropriate fields, some low-level inflammation and tissue redness can often result from the application of pulses. In the case of vaccination, this may provide a benefit (Babiuk et al., 2004). Upon electroporation in muscle or skin, low-level production and release of a number of different cytokines, including MIP-1, IP-10, and MCP-2, has been noted (Peng, Zhao, Xu, & Xu, 2007). These in turn cause the recruitment of neutrophils, monocytes, B cells, CD4+ and CD8+ T cells, and dendritic cells into the tissue (Babiuk et al., 2004; Liu, Kjeken, Mathiesen, & Barouch, 2008). While not enough to induce damage to the tissue, these chemokines and infiltrating cells may help to set up the perfect environment for enhanced immune priming.

A large number of antigens have been expressed for a variety of bacteria, parasites, and viruses in the development of electroporation-mediated DNA vaccines. Notable targets include *Bacillus anthracis* (anthrax) (Luxembourg et al., 2008), Hepatitis B virus (Luxembourg, Hannaman, Ellefsen, Nakamura, & Bernard, 2006; Zhao & Xu, 2008), Hepatitis C virus (Capone et al., 2006; Weiland et al., 2013; Zucchelli et al., 2000), Herpes virus (Babiuk et al., 2002), HIV (Babiuk et al., 2004; Cristillo et al., 2008; Liu et al., 2008; Widera et al., 2000), Influenza (Chen, Fang, Li, Chang, & Chen, 2005; Kadowaki et al., 2000; Ogunremi et al., 2013; Qiu et al., 2006), *Mycobacterium tuberculosis* (Tollefsen et al., 2002; Zhang et al., 2007), and *Plasmodium falciparum* (malaria) (Ferraro et al., 2013; Kumar et al., 2013; LeBlanc, Vasquez, Hannaman, & Kumar, 2008). One problem hindering DNA vaccine technologies has been that in many studies, success is seen in small animal models, most commonly mice, but there have been issues with translation to larger species, such as pigs or nonhuman primates. However, successes have been found and have led to several clinical trials for electroporation-mediated DNA vaccines, including Phase I trials for HIV (Vasan et al., 2011). In the case of the Phase I trial for HIV, expression of HIV-1 gag, pol, env, nef, and tat elicited an improved HIV-specific cell-mediated immune response, but not a humoral one, compared to DNA vaccination alone.

Another application of electroporation-mediated gene delivery into skeletal muscle has been the use muscles as bioreactors for the systemic delivery of therapeutic proteins. Levels of gene expression following electroporation-mediated delivery into the tibialis muscle can exceed 1 μg of luciferase in the mouse by 2 days and expression can persist for greater than a year, making this an ideal tissue to exploit as a bioreactor (Hojman, Gissel, & Gehl, 2007). The protein that has seen the most activity in this area is erythropoietin (EPO) for anemia and β-thalassemia (Maruyama et al., 2001; Payen, Bettan, Rouyer-Fessard, Beuzard, & Scherman, 2001; Rizzuto et al., 2000; Samakoglu et al., 2001). One elegant study focused on EPO delivery found that optimization of electroporation parameters, plasmid design, and dose could give long-term (>9 months) correction of hemoglobin and hematocrit to levels that could alleviate clinical manifestations of anemia (Hojman et al., 2007). Further, this study used a novel tetracycline-on promoter to drive EPO expression such that in the presence of doxycycline, EPO expression from the plasmid was turned on. Using this plasmid, Gehl and colleagues demonstrated that EPO and the corresponding levels of hemoglobin could be tightly controlled by varying the dose and duration of doxycycline administration. Thus, not only was the muscle being used as a highly efficient bioreactor, but also as one with a sensitive rheostat.

8. ELECTROPORATION OF SKIN: VACCINATION, WOUND HEALING, AND CANCER

The skin of an average adult covers approximately 20,000 cm^2 and as such, provides an ample target for gene delivery. As a consequence, like skeletal muscle, the skin may be a suitable organ for large-scale protein production. Moreover, the skin contains a large number of dendritic and Langerhans cells for antigen presentation, making it an excellent target for vaccine production. Indeed, Timitrov carried out the first *in vivo* electroporation-mediated gene transfer by delivering the neomycin resistance gene into the skin of newborn mice and subsequently isolating dermal fibroblasts to demonstrate transfer (Titomirov et al., 1991). Since then, a number of different approaches have been developed for the delivery of DNA and application of the electric field to this organ. One of the greatest goals for researchers, clinicians, and pharmaceutical companies is to develop as noninvasive and painless a method for this as possible. However, in almost all cases, the delivery of DNA itself into the skin raises problems. Since the

outermost layer of the skin, the stratum corneum, is essentially a layer of flat, keratin-rich dead cells and makes up a formidable impermeable barrier to the cells of the epidermis and dermis underneath, noninvasive delivery of DNA is largely impossible. Thus, for DNA to be delivered to the living cells of the skin, it must be injected directly into the tissue. As for skeletal muscle, the majority of electrodes fall into two categories: penetrating needle electrodes and parallel plate electrodes. In both cases, the electrodes are placed surrounding the site of DNA injection. While needles can be injected into the tissue, the plate electrodes usually pinch an area of skin around the injection site. Both electrode types give good gene transfer (Gilbert et al., 1997; Heller et al., 2007). More recently, the noninvasive MEA developed by Heller has used nonpenetrating needles to apply the field between electrodes that are at a very short distance from one another so that they deliver a low voltage to the tissue, thus reducing pain and increasing control of delivery (Donate et al., 2011; Ferraro et al., 2011; Guo et al., 2011; Heller et al., 2010).

Duration of expression in the skin is less than that seen in skeletal muscle, with expression high within 1–2 days then dropping off between 1 and 2 weeks, depending on the study (Medi, Hoselton, Marepalli, & Singh, 2005; Pavselj & Preat, 2005; Zhang, Nolan, Kreitschitz, & Rabussay, 2002). In order to increase this duration, it has been shown that injections and electroporations of the same plasmid can be done every few days to maintain and prolong expression (Heller, Jaroszeski, Coppola, & Heller, 2008). The distribution of gene expression is also variable, depending on the field and how it is applied as well as how DNA is injected. In several studies using the MEA, gene expression is first detected in cells in the epidermis within 2 days, but by 7 days, these cells were moving into the stratum corneum (Guo et al., 2011). As for other tissues, multiple "optimal" pulse conditions have been found that mediate high-level expression, but these vary based on the electrode type used and the preference of the investigators. Using the MEA, robust expression is seen when fields of 250 V/cm and 150 ms pulses are used (Guo et al., 2011).

Several studies have been undertaken to develop electroporation-based gene therapies to enhance wound healing in the skin. The first used the MEA to deliver plasmids expressing VEGF in an ischemic rat skin flap model (Ferraro, Cruz, Coppola, & Heller, 2009). Electroporation significantly increased VEGF expression in the skin for 5 days and resulted in upregulated endothelial nitric oxide synthase, increased perfusion in the skin flap, and

greatly improved wound healing. Another study expressed the LL-37 host defense peptide in full-thickness skin wounds using electroporation and showed that VEGF and IL-6 were upregulated and healing of the wounds improved over controls (Steinstraesser et al., 2014). Taken together, this is a new area that could have great clinical impact.

Since the skin contains an abundant number of professional antigen-presenting cells and is poised as a first line of defense for incoming antigens, its use as a site for vaccine production makes excellent sense. Indeed, a number of studies have expressed various antigens in the skin to take advantage of this high concentration of antigen-presenting cells. As in muscle, expression of antigens following DNA injection and electroporation generates both CD8+ and CD4+ responses (Brave, Nystrom, Roos, & Applequist, 2011; Glasspool-Malone, Somiari, Drabick, & Malone, 2000). An early study in the area by Préat and colleagues optimized expression of and immune response to luciferase in the skin following intradermal injection and electroporation and compared this to expression from muscle (Vandermeulen et al., 2007). While muscle was far superior to skin in terms of expression levels and immune responses generated, IFN-γ secretion by luciferase-stimulated splenocytes suggested that an efficient Th1 response was induced by both delivery routes. This group has also investigated the use of cell-specific promoters for expression in keratinocytes or dendritic cells and found that while the cell-specific promoters were able to drive low-level gene expression, they were unable to mount clear immune responses whereas general promoters that are ubiquitously active and strong (such as the cytomegalovirus (CMV) promoter) generated clear humoral and cellular immune responses, suggesting that generation of the immune response is dependent on the levels of antigen produced (Vandermeulen et al., 2009). As discussed above, the need for "painless" electroporation in the clinic has driven the development of needleless injection systems and nonpenetrating electrodes. The MEA developed by Heller has been used successfully to deliver the hepatitis B surface antigen in guinea pigs, an excellent model for skin vaccination due to its close resemblance to human skin. In these studies, robust expression of hepatitis B surface antigen was detected, as was minor inflammation and significant production of antibodies (Donate et al., 2011). A similar electrode was subsequently developed for use following intradermal DNA injection and has also been shown to elicit cellular and humoral responses against influenza NP antigen in mice, guinea pigs, and macaques (Broderick et al., 2011; Lin et al., 2011).

Electroporation-mediated gene delivery into the skin has also shown extreme promise in production of cytokines, receptor antibodies, and other agents to control cancer. While many of these approaches have been termed "vaccines" for cancer, they are not true vaccines since they are not generating immunity against the causative agent of the cancer (such as against HPV18 for cervical carcinoma), although they do provide protection from subsequent tumor challenge. For example, in one study in hamsters, the HER2 receptor was expressed in skin via electroporation and subsequently, the animals were challenged with HER2 positive carcinogen-induced oral carcinoma cells. Thirty-seven percent of animals that received the HER2 gene developed neoplasia compared to 74% of control animals (Berta et al., 2005).

Perhaps the best developed and most promising candidate for a cancer treatment using intradermal electroporation (or perhaps any type of electroporation) is the delivery of plasmids expressing IL-12 for the prevention, control, and treatment of metastatic melanoma. In 2002, Lucas and Heller demonstrated that expression of IL-12 in mice that had established B16.F10 melanoma tumors cured almost 50% of the mice of the tumor and caused 70% of these mice to be resistant to future challenge with B16.F10 cells (Lucas et al., 2002). Surprisingly, these results were seen only with intratumoral (e.g., intradermal) injection/electroporation of the plasmids but not following intramuscular injection/electroporation of the plasmids, despite robust expression of IL-12 following both treatment regimens. Subsequent studies show that these mice were essentially cured of the tumors for at least 100 days and that the treatment/protection provided by intradermal/intratumoral injection/electroporation of the IL-12 plasmid also provided treatment of and protection from tumors at distant sites as well (Lucas & Heller, 2003). These studies culminated in a Phase I first-in-man clinical trial, published in 2008 (Daud et al., 2008). Intratumoral injection and electroporation of the IL-12 plasmids caused marked tumor necrosis and lymphocytic infiltrates in a dose-dependent manner. Two out of nineteen patients with distant lesions that were not electroporated showed complete regression of metastases and the disease stabilized or showed partial response in an additional eight patients (Daud et al., 2008). These results suggest that intradermal/intratumoral DNA electroporation is a powerful tool for gene transfer and the treatment of metastatic cancer. A more detailed discussion of these studies and other clinical trials underway is presented elsewhere in this book.

9. ELECTROPORATION OF THE CARDIOVASCULAR SYSTEM

The vasculature presents a challenge to electroporation-mediated gene delivery using standard approaches. With the exception of a few large vessels, such as the ascending aorta, the walls of arteries and veins are too thin to be injected with DNA. Moreover, even if DNA could be delivered to the walls by injection, the architecture of the vessel wall would prevent the even distribution of DNA throughout it. Indeed, with multiple elastic laminas in large vessels and the tubelike structure of the vessel, injected DNA cannot freely diffuse throughout the wall. Consequently, in order to deliver genes to the vascular wall, addition of DNA can be from the outside or the inside of the vessel. For liposome and polymer-mediated delivery, intravascular injection is a simple and highly effective way to target the vascular endothelium, with the majority of gene transfer occurring in regions where the complexes get stuck due to reduced vessel diameter (e.g., the lung microvasculature). Alternatively, by injecting defined vessels that feed only one organ, transfer of DNA to that organ such as the kidney or liver or its proximal vasculature can also be achieved (Kobayashi et al., 2003; Tsujie, Isaka, Nakamura, Imai, & Hori, 2001). However, due to rapid flow rates, intravascular injection of DNA followed by electroporation has not been able to be used as a means to transfect vascular tissue, other than that in the liver or tumors (in this case, the electrodes are placed on the liver following intravascular injection of DNA) (Jaichandran et al., 2006). Various groups have transferred genes to the vasculature from within the lumen (Gunnett & Heistad, 2002; Matsumoto et al., 2001; Miyahara et al., 2006; Young & Dean, 2002). The advantage of this method is that with the appropriate device (e.g., double-balloon catheter or transient ligation; Figure 4), vectors can be delivered to defined regions of the vasculature with relatively simple methods that are clinically routine. The disadvantages are that in order for vectors to be delivered to the vessel lumen using double-balloon catheters, blood flow must be restricted, which may cause ischemia of the downstream vessels and tissues.

Alternatively, vectors can be administered from the adventitial surface of the vessel. The advantage of this approach of targeting defined regions of the vessel is that blood flow is not restricted, allowing no potential for ischemic injury. However, the major disadvantage is that the region of the vessel to be targeted for gene delivery must be exposed, requiring surgery. Our laboratory developed an electrode system to deliver DNA to the vessel wall from the adventitial surface that resembled a spoon containing

two parallel wires as electrodes that flanked a vessel segment that was positioned in the spoon (Figure 4) (Martin et al., 2000; Shirasawa, Rutland, Young, Dean, & Joseph, 2003; Young, Benoit, & Dean, 2003, Young, Zimmer, & Dean, 2008). Not only has this method been used to transfer DNA to vessels, but when catalytic oligodeoxynucleotides (DNAzymes) are added instead of plasmid, significant uptake of the oligonucleotides was detected along with detectable physiological activity (Nunamaker, Zhang, Shirasawa, Benoit, & Dean, 2003).

Most of the studies using this electrode system used the rat mesenteric vasculature as their target tissue. Instead of using an isolated vessel that has been dissected away from all adventitial tissue, we targeted the entire neurovascular bundle containing artery, vein, nerve, adventitial tissue, fat, and other tissue by laying it into the electrode, bathing it in the DNA solution (in 10 mM Tris, pH 8, 1 mM EDTA, and 140 mM NaCl), and applying a series of square-wave electric pulses. We found that 10 ms pulses at 200 V/cm gave very good expression that was sufficient to induce desired transgene responses (Shirasawa et al., 2003; Young et al., 2003, 2008). Using these parameters, an average of 400 pg of luciferase per centimeter of vessel (with a maximum of 2 ng per centimeter of vessel) was obtained 2 days post electroporation using 2 mg/ml DNA. When the field was raised to 400 V/cm, expression levels dropped off dramatically, likely due in part to tissue damage that was seen at this high field strength (Martin et al., 2000). This electrode system has been very successful in gene transfer to mouse and rat mesenteric vessels, the rat carotid, and mouse and rat femoral arteries. Moreover, by increasing the size of the electrode to accommodate larger tissues, we have also been able to successfully transfer genes without damage to segments of the mouse intestine itself.

The other main target organ in the cardiovascular system is the heart. Heller and colleagues first demonstrated that the porcine heart could be effectively electroporated by first injecting DNA into the myocardium and then applying asynchronous pulses to the heart using a penetrating four-needle array (Marshall et al., 2010). Since this study injected the myocardium directly and used electrodes that penetrated the heart, a thoracotomy was required to gain access to the heart. Electroporation using a field of 100 V/cm and 250 ms pulses increased gene delivery and expression of luciferase and VEGF by 25- and 5-fold, respectively, at 48 h compared to DNA injection alone. Although all animals survived following the application of these asynchronous pulses, all of the pigs showed electroporation-induced ventricular fibrillation that had to be treated by defibrillation. When

synchronous pulses were applied within the QRS wave, maximal expression of luciferase was seen using a field of 120 V/cm and 20 ms pulses with 15-fold higher expression detected at day 7, compared to DNA alone, and VEGF expression with synchronous pulses was 6-fold higher with electroporation. The need to administer the synchronized pulses within the QRS wave necessitated the shorter pulse lengths, thus perhaps dampening expression levels. Most importantly, using synchronized pulses, no ventricular fibrillation was detected. Subsequent studies testing different electrode configurations found that multiple electrode configurations (with varying depths of tissue penetration) gave significantly high gene expression compared to DNA alone and resulted in the distribution of gene delivery and expression throughout the myocardium at the sites of injection (Hargrave et al., 2013).

Another study in rats delivered DNA into the occluded coronary artery prior to application of the electric field using two-plate electrodes placed directly on the heart following thoracotomy (Ayuni et al., 2010). The field applied was 200 V/cm with a train of eight, 20 ms pulses. In these experiments, the pulses were applied asynchronously and resulted in no ventricular fibrillation, although a very transient asystole followed the electroporation in several of the animals that corrected itself without intervention.

Two additional studies have used electroporation-mediated gene delivery into the hearts of dogs to study the mechanisms of vagal-induced atrial fibrillation and to develop a therapy. Arora and colleagues demonstrated that atrial-selective attenuation of vagal signaling can be achieved by a specific G-protein C-terminal peptide delivered to the posterior left atrium (Aistrup et al., 2009). To develop a treatment method for this disease based on these findings, plasmids encoding these G-protein peptides were injected at multiple sites in posterior left atrium and a series of eight pulses (150 V/cm pulses at 20 ms each) were applied using two nonpenetrating needles (Aistrup et al., 2011). Not only were the transgenes expressed at the mRNA and protein levels, but they demonstrated physiological effects and were able to abrogate vagal-induced atrial fibrillation. Taken together, these studies show that electroporation has the ability to transfer genes safely to the heart and vasculature for a therapeutic outcome.

10. GENE DELIVERY INTO THE LUNG USING ELECTROPORATION

A number of viral and nonviral methods of gene delivery into the lung have been developed, but many have had limitations. The two most notable

limitations are the fact that many delivery approaches, especially viral methods, result in significant inflammation and that all methods result in the delivery of genes only into either the pulmonary epithelium (if delivery is via the airways) or the pulmonary endothelium (if delivery is intravascular). Since many lung diseases are either the result of, or are exacerbated by inflammation (e.g., asthma, acute lung injury, pulmonary fibrosis, etc.), techniques that increase local or systemic inflammation are undesirable. Since electroporation has the ability to drive DNA into different tissue layers, likely through its electrophoretic component, and shows very little inflammatory response in other tissues, it is an excellent choice for gene delivery into the lung.

We developed a simple method of electroporation-mediated gene delivery into the lungs: DNA is suspended in saline and delivered to the lungs by aspiration followed by electroporation using electrodes placed on either side of the chest (Dean, Machado-Aranda, Blair-Parks, Yeldandi, & Young, 2003; Machado-Aranda et al., 2005; Young, Barravecchia, & Dean, 2014). The major benefit of this approach is that it is very fast and simple: animals are lightly anesthetized, held in a position resembling a standing human, and the tongue of the animal is pulled out of the mouth with a pair of forceps. The DNA solution (50–100 μl for mice) is then delivered into the mouth, so the animals aspirate the solution into the lungs. If a finger is placed over the nares, faster and more robust aspiration is achieved. After the solution has been aspirated, the animals are returned to a supine position and allowed to recover. Once DNA has been delivered to the lungs, the animals are allowed to regain a normal breathing pattern (usually 15–30 s) and a series of electric pulses are applied to the chest. Flat electrodes, such as disposable, conformable, pediatric pacemaker electrodes, are placed on either side of the chest, usually under the armpits of the animals. A series of square-wave electric pulses are then applied across the chest, which causes the animal to jump slightly, unless paralytics have been administered (Emr et al., in press). Following electroporation, the animals are placed on their side and allowed to recover. The animals recover and survive with no apparent trauma until the experiments are terminated at the desired times, typically between 1 and 21 days post treatment.

We have found that gene transfer and expression are dependent on DNA dose with 10 μg of a CMV promoter-driven, luciferase-expressing plasmid giving about 2 pg of gene product per lung, whereas administration of 40 or 100 μg of plasmid yields 20 pg and 2 ng per lung, respectively, 2 days after electroporation (Dean et al., 2003). The duration of gene expression was

dependent on the promoter used in the plasmids: while the CMV immediate-early promoter/enhancer drove robust gene expression for up to 4–5 days in mouse and rat lungs with detectable gene expression appearing as early as 6 h, the human ubiquitin C promoter gave sustained gene expression for up to 6 months (Dean et al., 2003; Machado-Aranda et al., 2005; Pringle et al., 2007). As in other tissues, gene transfer is dependent also on field strength with a high level of expression being seen using trains of eight pulses of between 10 and 20 ms duration each at a field strength of 200 V/cm in mice, rats, and pigs (Dean et al., 2003; Emr et al., in press; Machado-Aranda et al., 2005). Unlike reports from others (Pringle et al., 2007), we have found no gene expression in the lungs without application of electric field (Dean et al., 2003; Machado-Aranda et al., 2005). As seen in the vasculature (Martin et al., 2000), when the field was increased to 400 V/cm, tissue damage, typically hemorrhage or inflammation, was observed in lungs. However, at 200 V/cm, there was no increase in hemorrhage, infiltrating lymphocytes or other cells, pulmonary edema, or alveolar wall thickening compared to control, unelectroporated lungs (Dean et al., 2003). Further, there was no increase in IL-6 or IFN-γ levels in the bronchial alveolar lavage fluid of animals electroporated with or without DNA compared to naïve animals or those receiving DNA only, and only a slight increase in TNF-α levels in electroporated mice compared to DNA-only mice (Dean et al., 2003; Zhou, Norton, Zhang, & Dean, 2007). Since TLR9, the innate immunity sensor in unmethylated CpG motifs on plasmid DNA, resides in the endosomal compartment, if electroporation is driving the bulk of the DNA into the cytosol directly, the DNA would never activate this pathway. Indeed, when plasmids were electroporated into TLR9-expressing or TLR9-knock out cells, IL-8 (a downstream TLR9-activated gene) expression was not increased compared to controls (Zhou et al., 2007). By contrast, when cells were transfected with the same plasmid using liposomal complexes, a significant induction of IL-8 was measured in TLR9-expressing cells, but not in the TLR9-null cells.

The only drawback to this approach of pulmonary gene delivery is one of perception. The most common argument against transthoracic electroporation is that the application of an electric field across the chest may likely lead to cardiac arrhythmias, fibrillation, and dysfunction. In the mouse and rat, application of a 200V/cm field with 10 ms pulses delivers roughly 0.1 J of energy. This is very low and insufficient to alter cardiac electrical function. However, the argument goes, if the technique is to be scaled up for human application, the resulting energy would be much higher. We

have had no mortality due to electroporation alone (n = 30 mice and 38 rats) and less than 5% mortality due to drugs, surgery, endotracheal tube placement, or fluid delivery (n ≥ 1000 mice and 400 rats). In 40 kg pigs, the gap between electrodes across the chest is ~15 cm. We found the optimal pulsing parameters, in terms of safety and maximal gene expression, used eight, 150 μs pulses at between 130 and 150 V/cm. Using these conditions, we have seen no deaths (n = 90 treated animals), no cardiac abnormalities, and found that the energy used for electroporation was less than 4 J (0.1 J/kg). This is much less than that used in many pacing modalities in humans and/or pigs and is low enough not to cause any arrhythmias or arrest. Since Advanced Cardiovascular Life Support guidelines use 200–360 J for emergent cardioversion on an adult, and 50–360 J for synchronized cardioversion, this procedure is not unreasonable (Field et al., 2010; Link et al., 2010; Neumar et al., 2010). Further, not only was the total energy 50- to 75-fold less, but the current delivered to the animals was a fraction of that delivered during cardioversion. Thus, the procedure is safe and effective for gene delivery in animals that are roughly the size of humans.

Since electroporation yields high levels of gene expression with little, if any, inflammation or trauma to tissues at the appropriate field strengths, we used this technique to transfer plasmids expressing the rat Na^+/K^+-ATPase β1 subunit to mouse and rat lungs and measured alveolar fluid clearance 2 or 3 days later (Machado-Aranda et al., 2005; Mutlu et al., 2007). This protein mediates movement of Na^+ and K^+ across the alveolar epithelium, resulting in net fluid movement into the interstitium and circulation. Overexpression of this protein could aid in edema removal in lungs with acute lung injury or acute respiratory distress syndrome (ARDS). Transfer of the β1 subunit by electroporation caused an 80% increase in alveolar fluid clearance in isolated rat lungs and a 60% increase in mouse lungs, whereas no significant changes in the rates of fluid clearance were detected in lungs that received DNA without electroporation (DNA only) or an empty plasmid (Machado-Aranda et al., 2005; Mutlu et al., 2007). When applied to a model of acute lung injury using mice exposed to lipopolysaccharide (LPS), we demonstrated that gene transfer of Na^+/K^+-ATPase β1 subunit both protected from subsequent LPS-induced lung injury and reduced injury in a lung that had been injured with LPS prior to any gene delivery (Mutlu et al., 2007). More recently, another group has shown that transfer of the Na^+/K^+-ATPase β1 subunit to mouse lungs can also protect from acute lung injury in a contusion model of lung injury (Machado-Aranda, Suresh, Yu, & Raghavendran, 2012).

In recent studies, we have seen similar improvements in outcome in a model of severe sepsis-induced acute lung injury/ARDS in pigs (Emr et al., in press). In these experiments, we used a "two-hit" model consisting of peritoneal contamination with a fecal-blood clot mixture and intestinal ischemia-reperfusion. Following injury, the animals were maintained in an intensive care unit with round-the-clock medical support for 48 h using the accepted drugs and ventilation strategies that are the clinical standard of care for patients with ARDS. Without intervention, 50% of the animals survived to the 48 h end point. We found that electroporation-mediated gene transfer of the Na^+/K^+-ATPase β1 subunit 4 h after injury improved lung function, maintained healthy lung architecture, reduced lung inflammation, and improved survival compared to animals electroporated with an empty plasmid (Emr et al., in press). The lung injury that is induced in this model is perhaps the most representative of what is seen in the neonatal, pediatric, and adult intensive care units following sepsis-induced ARDS and provides the most stringent and clinically relevant model in which we can evaluate electroporation for gene transfer in the lungs.

Several other groups have also developed protocols of electroporation-mediated gene delivery in the lungs. Schmid and colleagues routinely perform a thoracotomy to expose the lungs for electroporation. DNA in saline is either delivered intranasally to the lungs prior to thoracotomy and electroporation or to one lobe of the lung using a catheter following thoracotomy (Pringle et al., 2007; Gazdhar et al., 2006, 2007, 2013). In both cases, the electric field is then applied to the left lung using plate electrodes on either side of the exposed lung (not the chest), a chest drain is placed into the hemithorax, and the chest is closed with sutures. As with transthoracic electroporation, gene delivery and expression are both DNA dose dependent and field strength dependent. When DNA is delivered to the entire lung and only the left lung is electroporated, there is between 100- and 1000-fold more expression seen in the electroporated lobes (Pringle et al., 2007; Gazdhar et al., 2006). Although a field strength of 800 V/cm using 2 ms pulses gave the highest levels of gene expression, there was significantly more lung injury at this strength than at a field of 200 V/cm using 20 ms pulses. Another recent study using this approach has shown that delivery of hepatocyte growth factor reduced bleomycin-induced pulmonary fibrosis in mice as assessed by histology, hydroxyproline determination, and design-based stereology compared with controls (Gazdhar et al., 2013). Electroporation has also been used to transfer the gene for keratinocyte growth factor (KGF) to lungs following pulmonary resection to aid in repair (Matsumoto

et al., 2009). It is well documented that the lung undergoes compensatory growth after injury or surgical resection in a number of animal models, and that levels of KGF are increased during this proliferation. When a KGF-expressing plasmid was electroporated into one lobe of the lung following removal of the other three lobes (a standard model for pneumonectomy), increased proliferation of cells and increased PCNA staining (a marker of proliferation) were detected in the lungs of animals receiving the KGF vector, but not in animals electroporated with the empty vector. Taken together, these studies show that invasive and noninvasive methods of pulmonary gene delivery are safe and can have profound benefit for lung diseases in preclinical models.

11. CONCLUSIONS

Of the physical methods of nonviral gene delivery, electroporation is the most developed and has had the greatest impact to date. The benefits of electroporation in gene delivery are evident: reduced inflammation and immune response, ease and relatively low cost of instrumentation, and increasing levels of gene transfer and expression, nearing and perhaps surpassing those of viral vectors. This ease of use, safety profile, and ability to generate high-level gene transfer and expression have evolved from use in isolated cells to applications in living tissues in animals and humans in just the past 30 years. Its use as a platform technology is boundless. This can be seen in the myriad of applications to various tissues, from solid tissues that can be readily injected with DNA and electroporated with simple needles to fragile internal organs that present different challenges for DNA administration and field application. Regardless of these potential difficulties, the fact that successful and safe gene delivery has been achieved in the laboratory and in human clinical trials points to the potential of this nonviral approach in multiple areas of gene therapy.

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CHAPTER FOUR

Hydrodynamic Delivery

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Contents

Abstract

Hydrodynamic delivery (HD) is a broadly used procedure for DNA and RNA delivery in rodents, serving as a powerful tool for gene/protein drug discovery, gene function analysis, target validation, and identification of elements in regulating gene expression *in vivo*. HD involves a pressurized injection of a large volume of solution into a vasculature. New procedures are being developed to satisfy the need for a safe and efficient gene delivery in clinic. Here, we summarize the fundamentals of HD, its applications, and future perspectives for clinical use.

1. FUNDAMENTALS OF HYDRODYNAMIC DELIVERY

Hydrodynamic delivery (HD) emerged in 1999 as an epoch-making nonviral method of overcoming a series of hurdles for *in vivo* gene delivery (Liu, Song, & Liu, 1999; Zhang, Budker, & Wolff, 1999). The original procedure is comprised of a mouse-tail vein injection, in which a plasmid solution in saline is injected over 5 s in a volume equal to 8–10% of body weight. The retro-orbital sinus was recently reported as an alternative route, especially in neonatal mice (Yan et al., 2012). Significant levels of transgene expression are seen in many internal organs including the lungs, heart, spleen, kidneys, and liver, among which the liver shows the highest level of gene expression with 40% hepatocytes transfected, compared to less than 0.1%

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of positive parenchymal cells in other organs, with a single injection of less than 50 μg of plasmid DNA (Liu et al., 1999). The major advantages of HD are its simplicity, convenience, and reproducibility. The procedure does not require special equipment or laborious preparations. Short specific training of personnel in this straightforward procedure is sufficient (Katsimpoulas, Zacharoulis, Habib, & Kostakis, 2012). Although irregularity of cardiac activity, significant liver expansion, and an increase in serum concentration of liver enzymes have been reported (Zhang, Ludtke et al., 2004), the electron cardiogram returns to normal after 90 s (Zhang, Ludtke et al., 2004), the expanded liver returns to its original size in 30 min (Suda, Gao, Stolz, & Liu, 2007), and blood concentrations of liver enzymes drop to normal ranges 72 h post-injection (Liu et al., 1999). Compared to methods of viral and nonviral vector-based gene delivery, HD has the least probability of inducing long-lasting adverse events or immunogenic reactions. Neither histological nor biochemical studies revealed prolonged tissue damage to animals when proper hydrodynamic parameters were employed.

Experimentally, a 27-gauge needle is commonly used in tail vein injections in mice (Liu et al., 1999), whereas 20–24 gauge needles are appropriate for rats depending on body weight (Zhou, Kamimura, Zhang, & Liu, 2010). Other sized needles can also be used depending on the injection volume and speed and the type of blood vessel receiving the injection. The volume administered through the tail vein to rodents is usually 80–100 ml/kg (Liu et al., 1999). For maximal delivery efficiency, mice should be injected as rapidly as possible, typically in 3–5 s (Liu et al., 1999). Instead, rats are typically injected at a rate of 2 ml/s using a power injector (Maruyama, Higuchi, Nishikawa, Kameda et al., 2002), although a handheld syringe can be used (Zhou et al., 2010). The isolated liver of the rabbit was injected at a rate of 15–20 ml/s, which can only be accomplished using a power injector (Eastman et al., 2002). The amount of DNA employed ranges from 0.1 to 10 mg/kg. An optimal dose is 3 mg/kg in rats (Maruyama, Higuchi, Nishikawa, Kameda et al., 2002) and 7 mg/kg in rabbits (Eastman et al., 2002). In mice, the plasmid DNA dose range is 0.5–2.5 mg/kg. While saline is the most commonly used vehicle for HD, other solutions have also been employed, including Ringers solution (Feng et al., 2004) and phosphate-buffered saline (McCaffrey, Ohashi et al., 2002).

Mechanistically, a large volume of DNA solution rapidly injected into the tail vein travels to the heart and induces cardiac congestion followed by rapid retrograde flow into the hepatic veins (Figure 1). The DNA solution reaches the sinusoids by pushing back blood preexisting in the

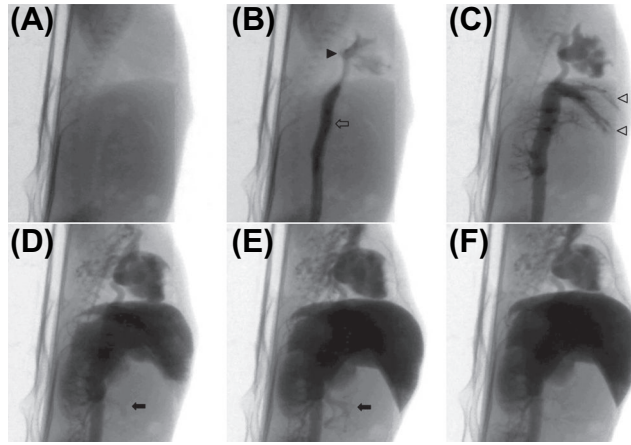

Figure 1 ***A continuous lateral view of fluoroscopic images recorded throughout the course of hydrodynamic injection.*** The contrast medium injected into the tail vein reached the heart first (B, closed arrowhead) via the inferior vena cava (B, open arrow), followed by the retrograde flow surging into the hepatic veins (C, open arrowheads). Although, the portal veins appear to be visualized after liver expansion (D, closed arrow) and gradually extended toward the periphery (E, closed arrow). As soon as the injection was completed, the portal vein could no longer be recognized (F). *Taken from Kanefuji et al. (2014).*

vasculature toward the portal side, preventing the quick mixing of DNA with nuclease-containing blood. The hemodynamic analysis of HD suggests that natural blood flow counteracts the hydrodynamically injected solution from the tail vein, allowing only a trace amount of spillover of injected solution into the portal vein (Kanefuji et al., 2014). Due to the tight junction holding the hepatocytes together and preventing pericellular passage of DNA solution, the hydrodynamic force expands the liver by stretching the vasculature, causing the enlargement of fenestra of the discontinuous endothelial lining lacking basement membrane, and forces invagination of the plasma membrane of the hepatocytes to allow DNA to move into hepatocytes (Suda & Liu, 2007). The unique structures of the liver, its location adjacent to the inferior vena cava (IVC), a large surface area of hepatocytes facing the lumen, and the high capacity of hepatocytes for gene expression are the primary reasons that a significantly higher transgene expression was seen in the liver compared to in other internal organs.

The expanded liver returns to its original size within 30 min as the cardiac functions are restored and the systemic homeostasis is reestablished. This is accompanied with a decrease in intravascular pressure and an increase in intracellular pressure of hepatocytes due to entry of DNA solution (Figure 2).

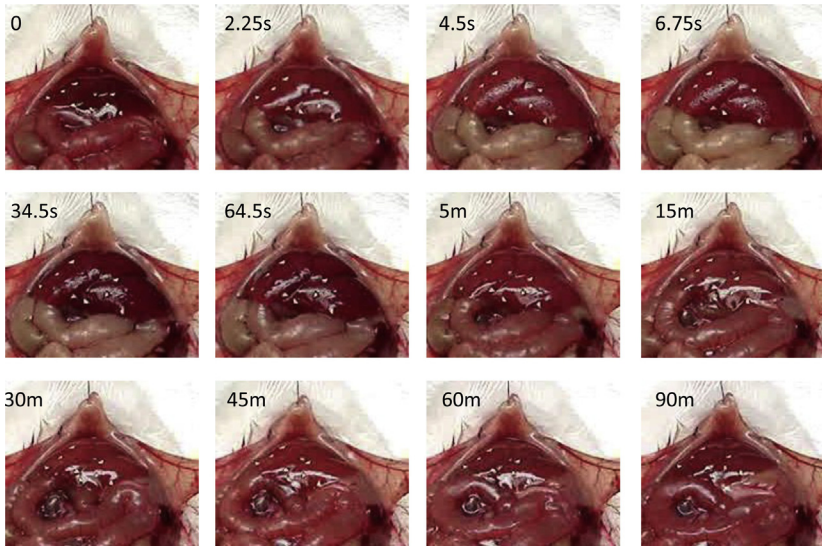

Figure 2 ***Impact of hydrodynamic injection on liver volume.*** Changes in volume and appearance of the liver were digitally captured through an open abdomen during and after the injection from the inferior vena cava in an anesthetized mouse, and representative snapshots are presented before (0), during (2.25 s), at the end of (4.5 s), and thereafter (6.75 s–90 m) the injection. The whitish triangular patches on the liver surface are markers attached for photogrammetry of the liver volume (Suda et al., 2007). The units "s" and "m" represent seconds and minutes, respectively. (For interpretation of the references to color in this figure see the color plate.)

The broken plasma membrane is resealed, and the endothelium regains its original structure in approximately 24 h (Zhang, Dong, Sawyer, Collins, & Fabre, 2004; Suda et al., 2007). When DNA solution is injected into the IVC and portal vein, the transfected cells in the liver are the hepatocytes located at the transition area of the zones 2 (Figure 3), where HD has the highest physical impact (Budker et al., 2006). The highest level of gene expression reported in mice by a single HD into a tail vein was 500 μg proteins per ml of serum (Zhang, Song, & Liu, 2000) and 45 μg of cellular protein per gram of the liver (Liu et al., 1999). It was shown that this level of gene expression was sufficient in restoring the function of blood coagulation in hemophilic mice (Miao, Thompson, Loeb, & Ye, 2001), establishing a mouse model of human viral infection (McCaffrey, Meuse, Karimi, Contag, & Kay, 2003; Yang, Althage, Chung, & Chisari, 2002; Wang, Contag, Ilves, Johnston, & Kaspar, 2005; Chang, Sigal, Lerro, & Taylor, 2001; Giladi et al., 2003), and eliminating the established tumor in tumor-bearing mice (Tada et al., 2006; Barnett et al., 2004; Takehara et al., 2007; Chen et al., 2003).

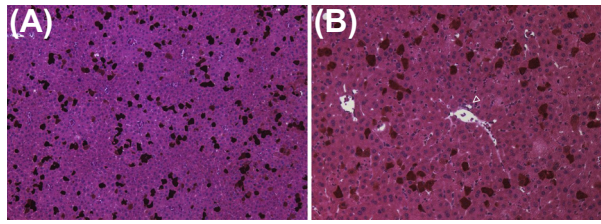

Figure 3 ***Distribution of transgene expression in mouse liver after hydrodynamic delivery (HD).*** Saline equal to 10% of body weight containing pCMV-luciferase plasmids was hydrodynamically injected either through the mouse-tail vein (A) or portal vein (B). The liver was harvested 24 h post injection and subjected to immunohistochemistry using an anti-luciferase antibody followed by hematoxylin–eosin staining. HD from the tail vein shows luciferase-positive hepatocytes surrounding the central vein (A), while positive hepatocytes encircling the portal area including the hepatic artery (arrowhead) in the case of HD via the portal vein (B). Nevertheless, luciferase-positive cells were located along the boundaries between zone 3 (one-third of acinus closest to central vein) or zone 1 (one-third of acinus closest to portal tract) and zone 2, respectively. Original magnifications are x100 in (A) and x200 in (B).

In contrast to earlier reports that HD involves DNA receptors (Budker et al., 2000; Kobayashi, Nishikawa, Hirata, & Takakura, 2004), the successful delivery of various compounds such as dyes, proteins, bacterial artificial chromosomes over 100 kbp, and even particles with approximately 6–8 μm in size suggests that HD is not a receptor-mediated process (Zhang, Ludtke et al., 2004; Suda et al., 2007; Kobayashi et al., 2004; Fumoto et al., 2009). Rapid elimination of preexpressed GFP in the cytosol (Kobayashi et al., 2004) using hydrodynamic injection, a quick recovery from a plasma alanine aminotransferase surge after the procedure (Suda, Suda, & Liu, 2008), transgene expression in hepatocyte culture isolated soon after HD (Kobayashi et al., 2004), and a transient window after HD allowing nucleic acids traverse cell membrane without hydrodynamic force (Kobayashi et al., 2004; Andrianaivo, Lecocq, Wattiaux-De, Wattiaux, & Jadot, 2004) support the notion that HD generates transient pores or membrane defects on hepatocytes to facilitate intracellular transfer.

In terms of the key determinant of HD, thorough investigations have suggested that injection speed, injection volume, static pressure in the liver, shear force of hydrodynamic flow of the injected solution, and liver volume at the end of injection are all important factors. A quick injection of a small volume induces high pressure, but delivery efficiency is not comparable to that achieved under the proper hydrodynamic condition (Liu et al., 1999). A large volume injection over 30 s instead of 5 s markedly reduces

delivery efficiency (Liu et al., 1999). In a fibrotic liver, the same injection profile gives rise to higher pressure leading to a stronger shear stress than in a normal liver with less transgene expression observed (Yeikilis et al., 2006). Furthermore, hydrodynamic or slow injection expanded the liver to the same degree as injection volume remained consistent (Figure 4) (Kenafuji et al., 2014), while gene delivery efficiency is different. Although many physical factors cannot be completely dissociated from each other in HD, organ expansion rate seems to be the most critical predictor for HD. Our recent work employing a computed tomography and contrast medium showed that the best condition for the liver is an expansion rate of 60%/5 s in mice (Kanefuji et al., 2014).

One advantage of employing HD in gene therapy studies is its low risk of inducing a host immune response compared with that seen in other

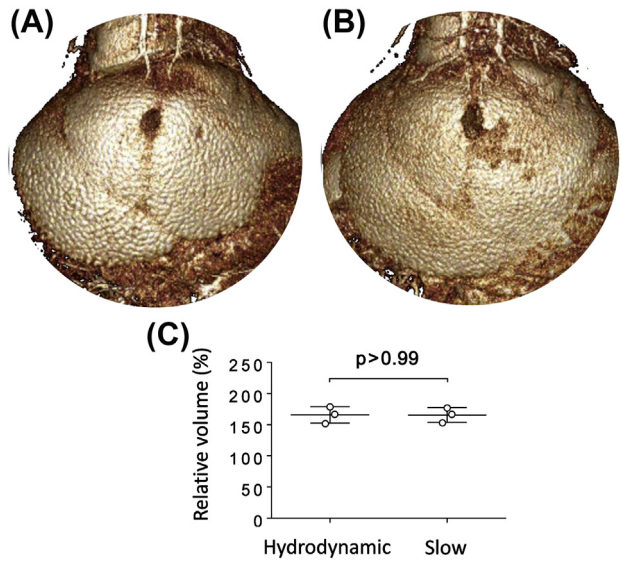

Figure 4 ***Liver volume difference between hydrodynamic and slow injections.*** Mice receiving hydrodynamic (A, 5 s) or slow (B, 60 s) injection of contrast medium equivalent to 9% body weight were subjected to a cone beam-computed tomography scan immediately after the conclusion of the injection, and maximum intensity projection images were reconstructed based on the three-dimensional volumetric data. The entire liver volumes were significantly enlarged after injection, but there were no significant differences between hydrodynamic (165.6 ± 13.3%) and slow (165.5 ± 11.9%) injections. The liver volume is expressed as a percentage of the volume from three control mice without injection in mean ± SD (C, $p > 0.99$, Mann Whitney test). *Figure 4(C) is taken from Kanefuli et al. (2014).* (For interpretation of the references to color in this figure see the color plate.)

methods. Since nonviral vectors other than HD attain a transgene expression below a therapeutic level (Nishikawa, Nakayama, Takahashi, Fukuhara, & Takakura, 2008), viral vectors are generally employed even it requires a laborious procedure for preparation, and often induces a host immune reaction against viral components. For example, upon AAV gene transfer to the liver in a hemophilia clinical trial, factor IX was once detected in two human subjects and declined during a period of weeks (Manno et al., 2006), likely as a consequence of immunological rejection of transduced hepatocytes mediated by AAV capsid-specific $CD8^+$ T cells (Mingozzi et al., 2007). As no additional component other than DNA and despite the reports that CpG sequence in DNA is capable of inducing immune response through Toll-like receptors, no immune response has been reported in animals after hydrodynamic gene delivery.

2. APPLICATIONS OF HYDRODYNAMIC GENE DELIVERY

Due to its physical nature, HD has been the method of choice in intrahepatic delivery of a variety of molecules including circular DNA (Liu et al., 1999; Zhang et al., 1999), DNA fragments (Magin-Lachmann et al., 2004), bacterial artificial chromosomes (Magin-Lachmann et al., 2004; Hibbitt et al., 2007), RNA (McCaffrey, Meuse et al., 2002; Giladi et al., 2003; Layzer et al., 2004; McCaffrey, Meuse et al., 2002), oligonucleotides (McCaffrey, Nakai et al., 2003), proteins (Zhang, Ludtke et al., 2004; Kobayashi, Kuramoto, Yamaoka, Hashida, & Takakura, 2001), polymers (Kobayashi et al., 2001), small compounds (Zhang, Ludtke et al., 2004; Kobayashi et al., 2004), viral vectors (Suda et al., 2007; Suda et al., 2008; Chuai et al., 2014; Qiao et al., 2009), and tumor cells (Li, Yao, & Liu, 2011). While HD through mouse tail veins delivers the gene of interest most effectively to the mouse liver, it has been explored for gene delivery into other tissues of different animal species (Suda et al., 2007; Herweijer & Wolff, 2007). Successful HD has been achieved in the kidneys (Maruyama, Higuchi, Nishikawa, Hirahara et al., 2002; Kameda et al., 2004; Romoren, Thu, & Evensen, 2004; Hamar et al., 2004), skeletal muscle (Budker, Zhang, Danko, Williams, & Wolff, 1998; Zhang, Budker, Williams, Subbotin, & Wolff, 2001; Hagstrom et al., 2004; Danialou et al., 2005; Zhang, Ludtke et al., 2004; Sato et al., 2003; Liang et al., 2004), myocardium (Mann et al., 1999), hepatocellular carcinoma (Tada et al., 2006), and brain tumor (Barnett et al., 2004). Its simplicity and versatility lead to a wide variety of applications of HD, including functional study of

Table 1 HD-based functional analysis of therapeutic genes

Disease	Therapeutic genes	Reference
Obesity/diabetes mellitus	Leptin	Jiang, Yamato, & Miyazaki, 2003
	IL-10	Gao et al., 2013
	Adiponectin	Fukushima et al., 2007
	Adiponectin receptor-2	Ma & Liu, 2013
	Uncoupling protein 1	Gonzalez-Muniesa, Milagro, Campion, & Martinez, 2006
	FGF21	Gao et al., 2014
	Protein tyrosine phosphatase 1B	Vakili et al., 2013
	Insulin	He et al., 2004
	IL-6	Mukumoto, Takahashi, Ando, Nishikawa, & Takakura, 2013
	Irisin	Bostrom et al., 2012
Myocarditis	IL-18-binding protein	Chang et al., 2014
	IL-1 receptor-II fusion gene	Liu et al., 2005
	IL-10-Ig	Higuchi et al., 2003
Cardiac disease	Mutant estrogen receptor	Chen et al., 2014
Nonalcoholic steatohepatitis	Inducible nitric oxide synthase	Anavi, Hahn-Obercyger, Margalit, Madar, & Tirosh, 2013
Hepatitis	HBV knockdown	Yang et a.l, 2002
	HCV knockdown	McCaffrey, Ohasi et al., 2002
Fulminant hepatitis	NKG2D knockdown	Huang, Sun, Wei, & Tian, 2013
	Osteopontin knockdown	Saito et al., 2007
Liver injury	c-Met	Zhu, Li, Li, Wu, & Gao, 2012
	IL-37	Bulau et al., 2011
	Caspase knockdown	Zender et al., 2003
Liver fibrosis	Platelet-derived growth factor receptor β knockdown	Chen et al., 2008
Hemophilia	Factor VIII, IX	Miao, Ye, & Thompson, 2003

Table 1 HD-based functional analysis of therapeutic genes—cont'd

Disease	Therapeutic genes	Reference
von Willebrand disease	von Willebrand factor	Rydz et al., 2013
	C-type lectin domain family 4 member M	Rydz et al., 2013
Osteoporosis	Insulin-like growth factor-1	Liu et al., 2013
Pancreatitis	Pancreatitis associated protein 1	Shigekawa et al., 2012
Muscular dystrophy	Dystrophin	Zhang et al., 2010
	miR-206	Guess, Barthel, Pugach, & Leinwand, 2013
Transdifferentiation	Pancreatic and duodenal homeobox 1, neurogenin 3, and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A	Cim et al., 2012
Regeneration	Fibroblast growth factor 7	Tsai & Wang, 2011
Autoimmune disease	FcgR-Ig	Shashidharamurthy et al., 2012
Renal injury	Hepatocyte growth factor	Bu et al., 2011
Renal fibrosis	Connective tissue growth factor knockdown	Yokoi et al., 2004
Glomerulonephritis	Viral IL-10	Higuchi et al., 2003
Arthritis	Chemotaxin 2	Okumura et al., 2008
Atopic dermatitis	IFN-γ	Watcharanurak et al., 2013
Bacterial infection	Kallistatin	Lu et al., 2013
Trypanosome infection	Trypanosome lytic factor	Thomson, Molina-Portela, Mott, Carrington, & Raper, 2009
Viral infection	Influenza A virus knockdown	Tompkins, Lo, Tumpey, & Epstein, 2004
Alpha-1 antitrypsin deficiency	α-1 antitrypsin	Zhang et al., 2000

(*Continued*)

Table 1 HD-based functional analysis of therapeutic genes—cont'd

Disease	Therapeutic genes	Reference
Growth hormone deficiency	Growth hormone	Sondergaard, Dagnaes-Hansen, Flyvbjerg, & Jensen, 2003
Hyperparathyroidism	Parathyroid hormone	Lee et al., 2008
Atherosclerosis	Nicotine acetylcholine receptor α1 knockdown	Zhang et al., 2011
Mucopolysaccharidosis-type VII	β-Glucuronidase	Richard, Arfi, Seguin, Gandolphe, & Scherman, 2009
Fabry disease	α-Galactosidase A	Nakamura et al., 2008
Metachromatic leukodystrophy	Arylsulfatase	Takakusaki, Hisayasu, Hirai, & Shimada, 2005
Mucopolysaccharidosis I	α-L-iduronidase	Camassola et al., 2005
Acyl-CoA dehydrogenase deficiency	Short-chain acryl-CoA dehydrogenase	Holm et al., 2003
Cancer	IL-15	Ochoa et al., 2012
Rejection after the transplantation	Endo-betagalactosidase C	Miki et al., 2004
Organophosphate toxicity	Paraoxonase	Fu, Wang, & Sun, 2005
Hypoxic-ischemia encephalopathy	Erythropoietin	Wang et al., 2004

HBV, hepatitis B virus; HCV, hepatitis C virus.
Taken from Kamimura (in press).

genes (Table 1), regulatory elements (Table 2), and the establishment of animal disease models (Table 3). In an effort to cope with the growing epidemics of obesity and obesity-associated metabolic disorders, genes involved in energy metabolism are also investigated (Gao et al., 2013; Gao, Ma, Cui, & Liu, 2014; Ma & Liu, 2013). HD allows researchers to validate the function of candidate genes using both knockin and knockout strategies to modulate functions of target genes for monogenic as well as multifactorial diseases.

The use of HD for protein drug discovery is another promising application. Conventional approach for protein drug discovery involves: (1) cloning of gene coding for the candidate protein into a bacterial expression cassette or eukaryotic expressing vector if yeast or mammalia cells will be

Table 2 Application for functional analysis of regulatory elements

Functional analysis of genetic element	Reference
Promoter sequence	Bonamassa, Ma, & Liu, 2012
Intron sequence	Wooddell, Reppen, Wolff, & Herweijer, 2008
CpG motif	Togashi, Harashima, & Kamiya, 2013
Integration elements	Umemoto et al., 2012
Transposon	Doherty et al., 2012
Scaffold/matrix attachment region	Wong, Argyros, Coutelle, & Harbottle, 2011
MicroRNA recognition elements	Wolff, Wolff, & Sebestyen, 2009
Cre-recombinase	Zhu, Wang, Feng, Sai, & Xue, 2006
Bacterial backbone	Zhu et al., 2009
Transcription factors for *in vivo* cell reprogramming	Yilmazer, de, Bussy, & Kostarelos, 2013
Enhancer	Taher, Smith, Kim, Ahituv, & Ovcharenko, 2013
Viral elements	Qiao et al., 2009
Cell-specific expression cassettes	Jacobs et al., 2008

used to express the gene; (2) introducing cloned gene into selected cells to produce the proteins in large-scale cell or bacterial culture; (3) preparing cell lyses and purifying protein using centrifugation, salt-based precipitation, and column chromatography; (4) determining protein purity and performing *in vitro* characterization; (5) assessing protein stability, bioavailability, formulation development, pharmacokinetics; (6) evaluating therapeutic activity and toxicity in disease-bearing animals; and then (7) conducting clinical evaluation. If HD is used, the cloned gene can be directly introduced into disease-carrying animals bypassing steps (1)–(5) for conventional approach, saving

Table 3 Application for establishing animal disease model

Disease model	Genes delivered	Reference
Hypertriglyceridemia	Lipoprotein lipase	Sun et al., 2013
Hepatitis B	Viral genome	Bloom, Ely, Mussolino, Cathomen, & Arbuthnot, 2013
Hepatitis C	Viral genome	Kim et al., 2009
Hepatitis D	Viral genome	Chang et al., 2001
Liver fibrosis	Transforming growth factor-beta1	Yang, Hung, Chang, & Li, 2007
Liver cancers	c-Met	Tward et al., 2007
Sepsis	siRNA	Wesche, Lomas, Perl, Chung, & Ayala, 2008

time, money, and efforts. As the liver is the major site for hydrodynamic gene delivery, genes encoding secretory proteins are preferred because once the proteins are made in the liver, they will enter the blood circulation and find their target located at different sites. Another advantage of the HD-based system is the possibility of screening a large number of gene-coding sequences to identify those coding for therapeutic proteins. The gene-coding sequences, once cloned into an expression cassette either as fragment or in plasmid form, can be hydrodynamically injected into disease-carrying animals one by one or as a group to screen out those genes that do not show pathological improvement, narrowing down further experiments to those sequences demonstrating therapeutic activities. Consequently, efforts can be focused on genes that code for proteins exhibiting desirable properties of pharmacokinetics and therapeutic efficacy. We predict that HD-based protein drug discovery will become a routine procedure for protein and/or gene drug discovery.

3. HYDRODYNAMIC DELIVERY IN LARGE ANIMALS—TOWARD CLINIC APPLICATION

Although HD is well tolerated in rodents, the impacts of the procedure on animals are significant. Immediately after injection, mice become immobile and manifest labored breathing for about 5 min. Rats react more severely and may stop breathing after the injection. An injection of 6000 ml of DNA solution is less than 10 s into the blood circulation for a man with body weight of 60 kg is medically unacceptable. Pathologically, when a large volume of solution is rapidly infused into noncompressive blood, the infused solution exudes into the interstitial space between cells and forms tissue edema. If this happens in the lungs, the solution accumulates in the interstitial air space and interferes the effective gas exchange, leading to pulmonary failure. Over extension of the heart muscle due to volume overload could also cause a reduction in the contractility and insufficient coronary circulation, leading to relative ischemia of the myocardium and malfunction of cardiac conduction system.

To avoid these potential problems and apply the principle of HD to disease treatment in clinic, we have developed a computer-controlled injection device for tissue-specific regional HD (Suda et al., 2008; Kamimura, Suda, Xu, Zhang, & Liu, 2009; Kamimura, Zhang, & Liu, 2010). The design combines intravascular insertion of a balloon catheter through the jugular vein into one of the hepatic veins and a computer-controlled injection (Figure 5).

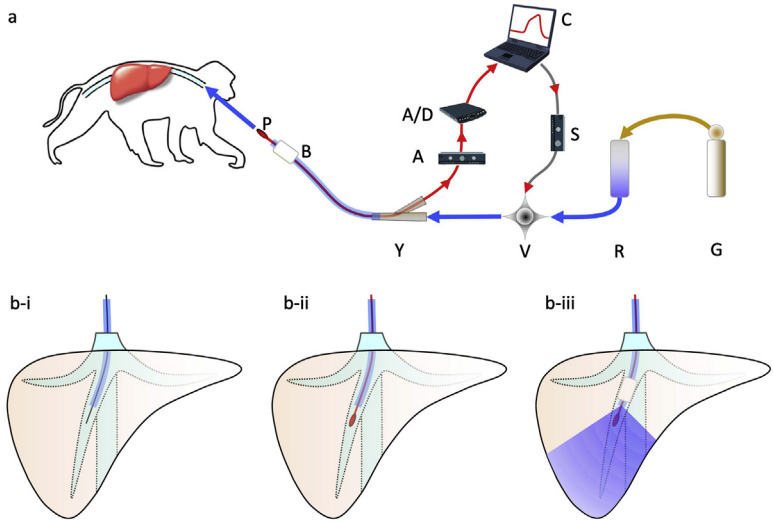

Figure 5 ***Schematic presentation of computer-controlled, image-guided hydrodynamic delivery to the liver.*** A balloon catheter is inserted under fluoroscopic guidance, and its tip is placed at a proper point in the hepatic vein (b–i). A pressure transducer (P) is coaxially inserted through a Y-shape connector (Y) (b–ii). Inflating the balloon (B), high-pressure CO_2 (G) propels DNA solution in a reservoir (R) to splash the solution into the target area (b–iii). The signal of elevated intravascular pressure is amplified (A) and sent to the computer (C) through an analogue/digital converter (A/D), controlling the power supply (S), and manipulating the valve (V) opening and closure to match the pressure with a preloaded profile. (For interpretation of the references to color in this figure see the color plate.)

The injection device consists of four major units: a pressure transducer, a solution driver, a computer, and an injection controller (Suda et al., 2008). A pressure transducer, located at the tip of the catheter, is inserted through a Y-shape connector into the balloon catheter in the targeted organ and sends the pressure data to the computer. In the first generation of the injection device, a pressurized CO_2 gas tank serves as the driving force in propelling the DNA solution into animal vasculature, and the injection is controlled by a solenoid valve (Suda et al., 2008), while an electromotor acts as both solution driver and injection controller in the second generation (Yokoo et al., 2013). The computer regulates the flow of DNA solution to reproduce a preset time–pressure curve upon a negative feedback circuit over the intravascular pressure.

Using a reporter construct, Kamimura et al. (2009) have demonstrated a significant level of reporter gene expression in the targeted liver lobe, as well as the safety and reproducibility of sequential injections in the same animal

(Kamimura et al., 2009). Importantly, due to direct injection into the hepatic vein, the volume required for optimal gene delivery has been reduced from about 10% body weight in rodents, to less than 5% in pigs (Suda et al., 2008). Regional HD can be used for gene delivery to organs other than the liver, such as the kidneys and muscle (Suda et al., 2008; Kamimura et al., 2010). Due to continuous endothelial structure, and in comparison with the fenestrae in the liver, a higher hydrodynamic pressure is required for successful gene delivery in these organs. Immunohistochemistry staining of the muscles revealed 70–90% of muscle cells expressing the reporter gene 60 days after HD (Kamimura et al., 2010).

Anatomically, hepatocytes are connected to, and approached by four different routes, including the hepatic artery, portal vein, bile duct, and hepatic vein. The injection via the hepatic artery or portal vein generates an injection flow in the same direction of the natural blood stream, which requires a blockade at the conjunction of IVC and hepatic veins in order to generate sufficient pressure increase (Zhang, Gao, et al., 2004). However, IVC occlusion creates a transient decrease in blood pressure and respiration rate followed by a rapid and a transient increase in heart rate upon the removal of the IVC occlusion (Kamimura et al., 2009). In addition, gene delivery efficiency did not show a significant increase, although the occlusion of blood flow in IVC or IVC with the portal vein was effective in elevating vascular pressure in HD through the hepatic vein (Kamimura et al., 2009). In addition, surgical intervention is required for needle insertion into the portal vein. A rapid injection and/or balloon occlusion at the hepatic artery could cause endothelial damage, leading to permanent vascular obstruction. To inject from the bile duct, a procedure successfully performed in rats, an endoscopic retrograde cholangiography that is known for potential complication including life-threatening pancreatitis, is required in humans. It was reported that the complication rate of endoscopic retrograde cholangiography is approximately 5% (Masci et al., 2001). Thus, regional HD through the hepatic vein is the most promising route in targeting the liver. Our recent work in assessing this procedure in dogs and baboons confirms that the image-guided, computer-assisted regional HD through the hepatic vein to the liver is safe and most desirable (Kamimura et al., unpublished data).

One significant finding from large animal studies is that the level of gene expression obtained is at least 100-fold lower than in mice (Kamimura et al., 2009). While efforts in improving efficiency are ongoing, the overall impact of hydrodynamic flow on the hepatocytes is of a major interest. When performed in rodents, the whole liver is impacted more homogeneously with

only the portal vein as the leaky site. In contrast, when regional HD is performed, the injected solution readily flows from the target area through physiological vascular connections of the meshwork of veins, arteries, portal veins, and each other, resulting in a lower impact on liver cells. In addition, varied gene delivery efficiency could be due to species differences. For example, liver compliance, as measured by surface indentation, is much lower in pigs when compared to these of mice, rats, dogs, and man (Carter, Frank, Davies, McLean, & Cuschieri, 2001). The higher content of fiber proteins in pig livers (Kamimura et al., 2009) and rodents with liver fibrosis (Yeikilis et al., 2006) reduced the effectiveness of HD. Other species-related differences such as the sinusoidal structure may also play an important role in determining the efficiency of HD. Although more studies in large animals are needed to identify factors that affect the hydrodynamic impact in favor of high gene delivery efficiency, it is likely that precise parameters for optimal HD in humans can only be defined in clinical trials.

4. FUTURE PROSPECTIVE

HD is one of the most effective methods for *in vivo* gene delivery. While the reliability of the procedure in rodents for gene functional studies and gene/protein drug discovery has been established, significant efforts are needed in defining the optimal parameters for clinical application. The most urgent issue to be resolved is to improve gene delivery efficiency in large animals. Studies focused on the identification of factors in large animals that interfere with gene transfer and expression are in great need before we can develop a new generation of injection devices for clinical evaluation of hydrodynamic gene therapy. The information generated from swine (Alino, Herrero, Noguera, Dasi, & Sanchez, 2007; Fabre et al., 2008; Yoshino, Hashizume, & Kobayashi, 2006; Kamimura et al., 2009; Kamimura et al., 2010), dogs (Kamimura et al., unpublished results), and isolated human liver (Herrero et al., 2012) appears critical for design of another set of experimentation that may pave the way for clinical application of HD.

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CHAPTER FIVE

Sustained Expression from DNA Vectors

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Contents

Abstract

DNA vectors have the potential to become powerful medical tools for treatment of human disease. The human body has, however, developed a range of defensive strategies

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to detect and silence foreign or misplaced DNA, which is more typically encountered during infection or chromosomal damage. A clinically relevant human gene therapy vector must overcome or avoid these protections whilst delivering sustained levels of therapeutic gene product without compromising the vitality of the recipient host. Many non-viral DNA vectors trigger these defense mechanisms and are subsequently destroyed or rendered silent. Thus, without modification or considered design, the clinical utility of a typical DNA vector is fundamentally limited due to the transient nature of its transgene expression. The development of safe and persistently expressing DNA vectors is a crucial prerequisite for its successful clinical application and subsequently remains, therefore, one of the main strategic tasks of non-viral gene therapy research.

In this chapter we will describe our current understanding of the mechanisms that can destroy or silence DNA vectors and discuss strategies, which have been utilized to improve their sustenance and the level and duration of their transgene expression.

1. INTRODUCTION

Typically, plasmid DNA (pDNA) vector-mediated transgene expression is transitory and reaches its maximum one or two days after administration to both cells in culture and to tissues in situ such as the liver or lungs. Following these peak levels of transgenic expression they subsequently drop swiftly and persist rarely beyond one week. Improving the genetic maintenance and the level and duration of gene expression from typical DNA vectors such as pDNA is essential to advance the field of nonviral gene therapy. First, it is important to understand why and how transgenic DNA might be silenced or destroyed.

Following gene delivery there may be several reasons for the loss of expression, such as the reduction in the copy number of pDNA itself by the removal of cells that have been lethally damaged during transfection, immune responses to cells expressing the transgene, and the removal of pDNA that has been recognized as foreign by the cell in the cytoplasm or the nucleus. The loss of expression may also be due to epigenetic events, where the pDNA is still present in the nucleus but expression is silenced and affected by positional effects of the chromatin status. By understanding the mechanisms that lead to the loss of expression, the design of gene delivery vectors can be developed rationally to extend and enhance their gene expression profile and their safety. In this chapter we will discuss the mechanisms which can lead to the loss of a DNA vector and the silencing of its expression and explore a variety of techniques which researchers have used to overcome them.

2. IMMUNE RESPONSES

The cationic polymers that are often used to formulate DNA vectors into nanoparticles and deliver them into cells are liable to activate the complement system (Plank, Mechtler, Szoka, & Wagner, 1996). Additionally, pDNA itself can elicit immunostimulatory responses. Bacterial DNA comprises relatively high numbers of CpGs (McLachlan, Stevenson, Davidson, & Porteous, 2000), compared with mammalian DNA, which are not methylated (McLachlan et al., 2000). The frequency of CpG motifs in vertebrate DNA is approximately 25–30% of that found in bacterial DNA and over 80% of these vertebrate CpGs are methylated. Dendritic cells, macrophages, and other antigen-presenting cells take up DNA complexes as well as the specifically targeted cells, eliciting maturation, differentiation, and proliferation of monocytes, macrophages, T-cells, and natural killer cells (Rothenfusser, Tuma, Endres, & Hartmann, 2002). Although nonviral DNA vectors are substantially less immunogenic that viruses, immune reactions still pose a hurdle for efficient gene transfer and expression. The conspicuous presence of unmethylated CpGs is detected by a family of protein receptors known as Toll-like receptors (TLRs), specifically TLR-9. TLRs are predominantly expressed in tissues involved in immune function, such as the spleen and peripheral blood leukocytes, and tissues exposed to the external environment, such as the lung and the gastrointestinal tract. TLR-9 was shown to activate transcription factors such as AP-1, NF-κB, and interferons (IFN) which induce a T helper 1-type spectrum of proinflammatory cytokines and chemokines, generating an innate immune response against the pDNA backbone which in turn leads to silencing of pDNA-encoded transgenes or elimination of transduced cells (Zhou, Liu, & Liang, 2004). Furthermore, the presence of expressed foreign proteins in the circulation can elicit antibody responses, again resulting in suppression of the expression of protein and removal of transgene-expressing cells (Sarukhan et al., 2001). In particular, there is evidence that suggests a cellular defense mechanism against foreign DNA which is integrated into the genome that results in the down-regulation of transgene expression (Baer, Schubeler, & Bode, 2000). This highlights the importance of understanding mechanisms of the immune system to achieve long-term gene expression *in vivo*.

Extensive studies by Kay et al. (Chen et al., 2001) demonstrated that the bacterial backbone of a pDNA construct is primarily responsible for the silencing of the vector. Separation of the expression cassette from the

bacterial backbone resulted in 10- to 100-fold higher expression in transgene levels compared with close circular DNA following hydrodynamic delivery. Methylation of CpGs in the bacterial backbone within nonpromoter sequences is a favored theory for inactivating expression. It is suggested that inactive chromatin structure spreads from these methylated regions leading to transcriptional inhibition (Kass, Goddard, & Adams, 1993). Other gene silencing mechanisms may also be responsible, such as chromatin structure, condensation state, and further involvement of methylated CpG-binding proteins.

3. PREVENTING EPIGENETIC SILENCING

Gene silencing is a natural process and is essential for the development of regulated gene control. During embryonic development, genes exhibit specific methylation patterns beginning with genome-wide demethylation after fertilization, rapid remethylation after the blastocyst implant in the womb, followed by gradual remethylation thereafter during development. Remethylation of the genome involves de novo methylation that occurs at discrete CpG sites due to the presence of *cis*-acting methylation signals. Once CpG sites within the *cis*-acting signals are methylated, they can act as sites for a second form of de novo methylation, which is spread to neighboring CpG sites.

Normal gene silencing is a balancing act between DNA methylation activities; open chromatin established by an active promoter and the spread of DNA methylation from existing inactive chromatin regions (Jenuwein & Allis, 2001). However, it is an event that occurs frequently following gene transfer into mammalian cells and is one of the factors that contribute to failure of transgene expression, especially with nonviral vectors. Eukaryotes have evolved mechanisms to maintain genome integrity and prevent expression of abnormal and foreign proteins. Transgenes generally lose expression over time, in part owing to de novo methylation by DNA methyltransferases. Most silencing processes are accompanied by CpG methylation, which is preceded by histone H3 methylation at Lys-9 (H3K9). Such methylation may in turn trigger chromatin condensation that spreads to a downstream promoter, creating a heterochromatin structure, especially in cases where the spread of methylation is not blocked by an insulator. This phenomenon is not limited to integrated transgenes and DNA methyltransferases has also been reported to act on episomally maintained expression, such as on transgene expression mediated by the Epstein–Barr virus (EBV) vector (Hsieh, 1999).

Nevertheless, DNA methylation is a reversible process and by changing the methylation status of DNA during development, gene expression can be temporally regulated (Cedar, 1988; Weiss & Cedar, 1997). It has become clear that cells display a specific pattern of DNA methylation that determines the properties of the cells (Ohgane, Yagi, & Shiota, 2008). Interestingly, progenitor cells have low levels of DNA methylation and therefore, may be less prone to gene silencing. However, these cells usually methylate portions of their chromosomes upon differentiation and may silence integrated transgenes together with endogenous genes. Therefore the employment of genetic elements that may counteract DNA methylation and silencing events may be of particular benefit for stem cell therapies. However, the extent to which the cellular DNA methylation profile may influence silencing of transgene in differentiated cells has yet to be elucidated.

4. POSITION EFFECT

The chromatin environment also influences the expression of the integrated transgene into the host chromosome in relation to its location, a phenomenon known as position effect. High-level expression can only be achieved at specific nuclear sites that are specialized in RNA synthesis and that are protected from nuclear regions where inactive genes are localized. Chromosome architecture and chromatin structure together ensure efficiency of gene expression at favorable genomic loci. During RNA synthesis, active RNA polymerase complexes and transcription factors associate with a "transcription factory" that influences the chromatin state. There appears to be a transition in chromatin loops by chromatin-remodeling and histone-modifying enzymes (Fiorini, Gouveia Fde, & Fernandez, 2006) to ensure the gene is localized close to the active transcription factors after which RNA synthesis proceeds. This correlates with the concept that cycles of gene activity and inactivity occur in bursts, as a majority of mammalian genes are transcribed infrequently and the dynamic changes of chromatin loop attachment places the gene within euchromatin and only then transcription can occur. The understanding of the structure and function of these transcription sites is important for the development of nonviral vectors to target these active chromatin regions and avoid compartments where gene expression is switched off. Such directed integration can be driven by specific enzymes endogenously present in viruses for infecting prokaryotes, called site-specific recombinases (SSR) that recognize unique nucleic acid sequences and recombination sites (RS), within the pathogen and host genomes. These SSRs are

able to mediate recombination at the specific sites, although with varying degrees of efficiencies. In eukaryotes, similar sites have been shown called pseudoRS (Voziyanov, Pathania, & Jayaram, 1999). Many bacteriophage SSRs have been manipulated for gene therapy, some of which require bacterially expressed cofactors such as IHF for recombination (including λ; Christ, Corona, & Droge, 2002), HK022 (Kolot, Silberstein, & Yagil, 1999), and HP1 (Esposito, Thrower, & Scocca, 2001) and some that are able to mediate integration in human cells without bacterial cofactors (including recombinases Cre, phiBT1 (Chen & Woo, 2008)), and phiC31 (Hollis et al., 2003).

However, there are concerns that the transgene integration into highly expressed sites may interfere with vital cellular functions. Furthermore, there remains the potential for random integration that may cause insertional mutagenesis.

5. PERSISTENT NONINTEGRATING EPISOMAL DNA VECTORS

Episomal vectors offer several advantages over integrating vectors by persisting in the nucleus in an extrachromosomal state. The first is that the gene of interest will not be disrupted or subject to regulatory constraints. Second, the use of episomal vectors will not lead to cell transformation because physical integration of the vector into the genome is avoided. Furthermore, episomal vectors persist in multiple copies per cell, allowing high expression of the gene of interest. In stable transfection procedures, the use of episomal vectors results in higher transfection efficiency than the conventional nonepisomal vectors, which integrate at a low frequency (Mazda, Satoh, Yasutomi, & Imanishi, 1997). Also, episomal vectors have a high insert capacity therefore allowing delivery of entire genomic DNA loci, which is proven to achieve physiological levels of transgene expression (Huang et al., 2000; Schiedner et al., 1998). The advantages of extrachromosomal constructs have led to increasing efforts to develop these vectors for gene therapy models and preclinical studies.

6. EPISOMAL DNA VECTORS BASED ON REPLICATION-DEFICIENT VIRUSES

Episomal viral vector systems have been developed based on replication-deficient virus-like particles (VLPs), such as AdVs and AAVs, where the virus vector is able to actively persist in the genome in a

nonintegrative state by utilizing viral proteins. Other viral episomal systems are based on viral plasmid replicons like the Simian Virus 40 (SV40), bovine papillomavirus, or the EBV. The difference between episomal systems based on VLPs and episomal systems based on viral plasmid replicons is that VLPs are assembled as virions in separate producer systems and these vectors are able to exhibit all viral functions for the transduction of target cells and nuclear translocation of their genomes. They only lack the ability of the respective wild-type virus to replicate and produce progeny. In contrast, viral plasmid replicons are not assembled as virions and therefore lack the ability to infect target cells. These vectors are transfected into target cells as naked DNA, but once in the nucleus the viral plasmid replicon vectors are able to exhibit efficient strategies for their DNA replication and nuclear retention (Ehrhardt et al., 2008). Nevertheless, in both cases, these modified viral vectors are expensive to produce and safety concerns remain. Leaky viral products have been reported using replication-deficient AdVs which induce host's immune responses. In addition, low-level integration and immunotoxicity against the viral capsid of replication-deficient AAVs have been shown at high multiplicities of infection (Nakai et al., 2003) and EBV plasmid replicon vectors has been shown to cause B-cell immortalization up to several 1000-fold (Humme et al., 2003) while stimulating both humoral and cellular immune responses (Taylor et al., 2004).

Episomal vector systems based on substitution of viral plasmid replicons mimic natural episomal viruses such as SV40 and EBV, which require viral proteins for their cellular replication machinery. For example, the SV40 virus requires the large T-antigen (Tag) (Li et al., 2003) while the EBV virus requires EBV nuclear antigen-1 (EBNA1) (Wu, Ceccarelli, & Frappier, 2000) for initiation of replication at the viral origin of replication (*ori*). The presence of the viral proteins is required for the segregation of the viral episomes to progeny cells by association with an origin of replication complex (ORC) mediating the so-called 'piggy-back' mechanism (White, Wade-Martins, & James, 2001) (Figure 1). Addition of the EBNA1 gene to a Factor IX (FIX)-encoding pDNA was found to increase FIX expression about a 10- to 100-fold in nondividing liver cells of mice (Sclimenti et al., 2003). The development of an EBV-based episomal pDNA was able to stably express hypoxanthine phosphoribosyl transferase (HPRT) at close to physiological levels for up to six months in HPRT-deficient human lung fibroblast cells (Wade-Martins, White, Kimura, Cook, & James, 2000). However, over time the EBV-based episomes were lost at a rate of 4% per cell division in the absence of antibiotic selection pressure while continuous selection

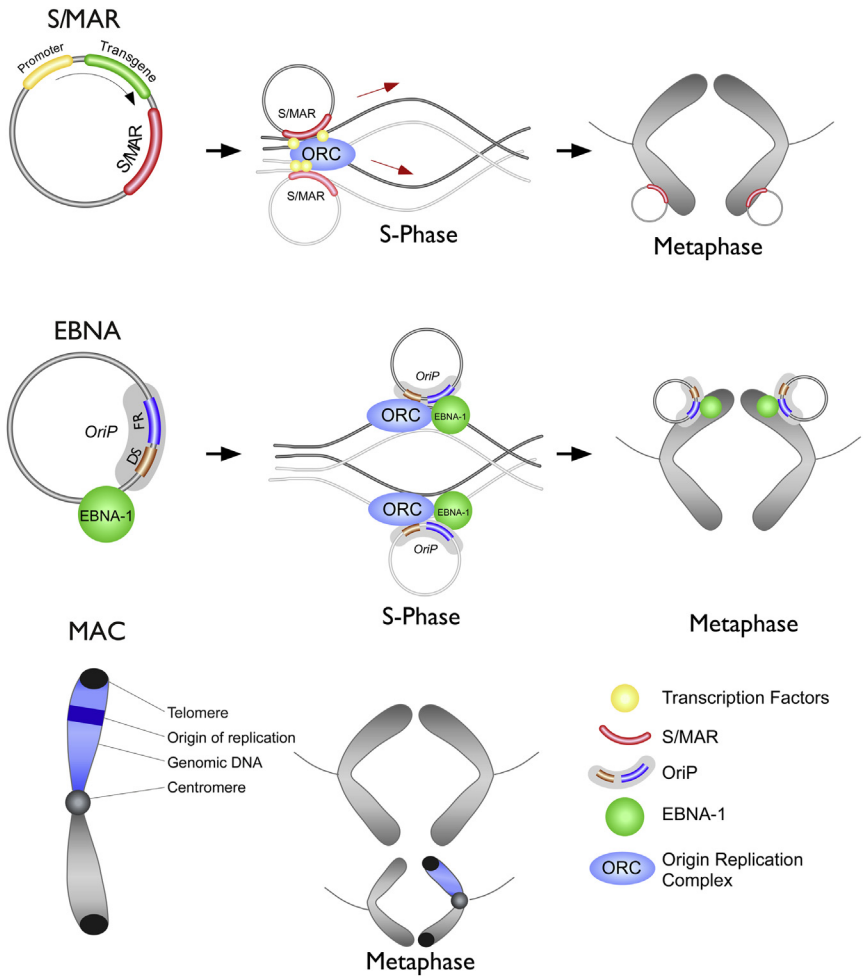

Figure 1 ***Schematic representation of the elements and mechanisms which mediate the episomal retention of DNA vectors.*** The different mechanisms of mitotic stability of S/MAR DNA vectors, EBV vectors, and MACs in replicating cells mediated by association with the chromosomes (EBV- and S/MAR-based vectors) or interaction with spindle fibers (MACs). Compared to EBV, the S/MAR DNA vector does not require viral proteins for its replication and segregation. DS, dyad symmetry; EBNA-1, EBV nuclear antigen-1; FR, family of repeat; oriP, origin of plasmid replication. (For interpretation of the references to color in this figure see the color plate.)

pressure led to integration of the plasmid into the host genome. Furthermore, Tag is shown to disrupt the retinoblastoma and p53 tumor-suppressor pathways and alter the function of other key host cell proteins leading to transformation (Ali, Kasper, Arai, & DeCaprio, 2004).

7. EPISOMAL DNA VECTORS BASED ON CHROMOSOMAL ELEMENTS

Due to safety concerns related to virus-based episomes and episomes harboring viral proteins, efforts have been focused on construction of episomally replicating vectors that are composed solely from functional chromosome elements. These elements are based on knowledge gleaned from studies into viral vectors that provide insights into the mechanisms by which levels and kinetics of expression can be optimized.

8. MAMMALIAN ARTIFICIAL CHROMOSOMES

One such design is the construction of vectors that resemble natural chromosomes. Artificial chromosomes would allow stable maintenance at a low and defined copy number in the cell in the absence of selection and have an infinite cloning capacity. Chromosomes have three main components: a centromere, telomeres at both termini, and *oris* (see Figure 1). The first human artificial chromosomes (HACs) produced were unstable and integration and disruption of the host genome was commonly observed (Harrington, Van Bokkelen, Mays, Gustashaw, & Willard, 1997), with the exception of the HAC created by Ebersole et al., where circular HACs were shown to be mitotically stable in the absence of selection (Ebersole et al., 2000). The large cloning capacity of mammalian artificial chromosomes (MACs) is attractive as it allows the therapeutic genes with their entire natural genomic content containing all distal regulatory elements to be delivered.

There are two approaches for the design of an HAC. In the "top–down" approach, natural chromosomes are reduced to a minimal length by irradiation or telomere fragmentation, resulting in minichromosomes (Farr, Stevanovic, Thomson, Goodfellow, & Cooke, 1992) whereas the bottom–up approach involves the combination of long synthetic arrays of α satellite DNA (a major component of natural human centromeres) with telomeric DNA and ~100-kb genomic DNA fragments. So far, most types of artificial chromosomes have been introduced into mice either by microcell-mediated chromosome transfer or pronuclear injection (Basu & Willard, 2005; Jackson, Juranek, & Lipps, 2006). A circular HAC containing the guanosine triphosphate cyclohydrolase I gene, and a linear HAC containing the human globin gene cluster, were introduced into mouse embryonic stem cells that were stably maintained for at least three months and used

to create genetically modified chimeric mice (Suzuki, Nishii, Okazaki, & Ikeno, 2006) whereas a human minichromosome from a hamster cell line was successfully transferred to a mouse embryonic stem cell line (Paulis et al., 2007). In another study, an *in vitro* assembled maize minichromosome was stably transmitted during meiosis (Carlson et al., 2007). More recently, however, infectious herpesvirus-based delivery systems for HACs have been constructed that enable the delivery of large (up to 100 kb) HAC inserts into a variety of target cells (Lufino, Manservigi, & Wade-Martins, 2007; Moralli, Simpson, Wade-Martins, & Monaco, 2006).

However, the production of the large-sized MACs at a scale sufficient for therapeutic benefit remains a challenge. Furthermore, there remain ethical and safety issues with MACs, as the source of the chromosomal elements such as telomeres, centromeres, and *ori*, has to be controlled stringently particularly if removed from animal cells.

9. EPISOMALLY MAINTAINED pDNA VECTORS

pDNA vectors transfected into cultured cells are generally rapidly lost during cell division unless selection pressure is applied. Selection driven by the application of toxic drugs may not only influence the molecular behavior of the cell, it may also occasionally result in the random integration of the DNA vector into the host genome. Integration can lead to deregulated expression or silencing of the vector as well as potentially damaging the host cell through the disruption of normal genomic expression patterns or by integrative mutagenesis. When DNA vectors are applied *in vivo* to nondividing or slowly dividing tissues (such as the liver and skeletal muscles), they tend to be passively maintained and are rapidly lost when the tissue is forced to undergo cellular division (such as following a partial hepatectomy) (Argyros et al., 2008; Jacobs et al., 2008).

Efforts have focused on identifying DNA elements that support episomal maintenance and replication in pDNA vectors in the absence of drug-mediated selection pressure. Such a vector called pEPI was generated in which the traditionally described viral proteins required for episomal maintenance were replaced by an element called a scaffold/matrix attachment region (S/MAR) from the human β interferon gene cluster (Piechaczek, Fetzer, Baiker, Bode, & Lipps, 1999).

This S/MAR pDNA vector was demonstrated to propagate episomally for several hundred divisions in every mammalian cell line tested such as CHO (Piechaczek et al., 1999), human cervical cancer HeLa

(Schaarschmidt, Baltin, Stehle, Lipps, & Knippers, 2004), pluripotent p19 embryonic carcinoma (Sotirova, Calciano, Krueger, & Lalande, 2006), human hematopoietic stem cells (Papapetrou, Ziros, Micheva, Zoumbos, & Athanassiadou, 2006), human hepatoma Huh7 (Argyros, Wong, Gowers, & Harbottle, 2012), and human lung epithelial IB3 cells (our own observations). The pDNA vector pEPI was also used to express a short-hairpin RNA against the *bcr-abl* gene, remaining in the human hematopoietic progenitor cell line K562 as an episome for a period of four months in the absence of selection (Jenke et al., 2005). In another study, sperm-mediated gene transfer of pEPI to oocytes resulted in strong marker gene expression in nine out of 12 pig fetuses (Manzini et al., 2006). Even though the fetuses were not brought to term, this study highlights the ability of S/MAR for providing stable efficient expression *in vivo*. In other studies, the inclusion of the S/MAR element in a pDNA was able to provide long-term regulated functional complementation of the low-density lipoprotein receptor (LDLR) (Lufino et al., 2007). Similarly by incorporation of S/MAR in a hFIX expression cassette, a five-fold increase in expression was observed compared to that of the vector without the S/MAR, and was sustained for a year (Ehrhardt, Peng, Xu, Meuse, & Kay, 2003). All the essential requirements for extrachromosomal stability appear to be provided by the S/MAR element.

10. SCAFFOLD/MATRIX ATTACHMENT REGIONS

S/MARs are defined as the genomic DNA sequences at which the chromatin is anchored to the nuclear matrix proteins during interphase (Mirkovitch, Mirault, & Laemmli, 1984). S/MARs mediate the attachment of chromatin to the scaffold forming looped domains that participate in various matrix-based processes involving DNA replication and transcription, RNA processing, as well as transport and signal transduction (Jackson & Cook, 1995). Although S/MARs are evolutionarily conserved, they have no consensus sequence but contain several recognizable motifs within and between species (Girod et al., 2007). Among these are replication initiator protein sites (ATTA), homo-oligonucleotide repeats, DNase I-hypersensitive sites, and potential nucleosome-free stretches (Boulikas, 1995). Evidence of the role of S/MARs in regulating gene expression comes from examination of S/MAR locations in long genomic DNA sequences that reveal S/MAR distribution is similar to the average gene density,

implying each gene has its own S/MAR—the so-called "one gene-one S/MAR" hypothesis (Frisch et al., 2002). Also, S/MARs appear to be free from interaction with the nuclear matrix in cells that do not express adjacent genes (de Belle, Cai, & Kohwi-Shigematsu, 1998) indicating that active transcriptional read-through is necessary for its activity. Furthermore, S/MARs are generally found close to enhancers and promoters in the first intron and the transcriptional effect depends on the distance from the promoter and on the direction of transcription (Boulikas, 1995). All these observations indicate that S/MARs play an active regulatory role in transcription rather than just a simple structural role of anchoring DNA to the nuclear matrix.

Notably, S/MARs also possess an A + T rich domain (over 70% AT) (Bode, Benham, Knopp, & Mielke, 2000; Mirkovitch et al., 1984) unlike regular enhancers and promoters, which is suggested to aid the unwinding and destabilization of DNA (Bode et al., 1992) or the formation of loops (von Kries, Buhrmester, & Stratling, 1991). The affinity of S/MARs for the nuclear matrix has implicated them in a variety of biological activities such as the insulation of transgenes from positional effects (Goetze et al., 2005), augmentation of transcription rates (Bode et al., 2000), long-term maintenance of high transcriptional levels (Argyros et al., 2008; Dang, Auten, & Plavec, 2000), as well as enhancer and origin of replication functions (Jenke et al., 2002; Lipps & Bode, 2001).

11. INSULATOR FUNCTION

The interaction between S/MARs and the nuclear matrix is important for the organization of chromosomal loops that define the boundaries of independent chromatin domains (Jenke et al., 2002) thereby insulating coding regions from the surroundings and establishing local access of transcription factors to promoters and enhancers. The function of S/MARs as insulators has been shown in studies demonstrating that S/MARs enhance expression of a reporter gene following integration in a cellular chromosome only in stably transfected cell lines, but not in transient transfection assays (Klehr, Schlake, Maass, & Bode, 1992; Stief, Winter, Stratling, & Sippel, 1989). This idea was reinforced by the "one gene-one S/MAR" hypothesis mentioned earlier that predicts that each active gene has its own S/MAR element (Bonifer, Vidal, Grosveld, & Sippel, 1990) and that S/MAR elements could effectively shield transgenes from position effects by creating

independent domains. In addition, the presence of S/MARs was shown to establish independent domains in transgenic mice completely restoring correct hormonal regulation (McKnight, Shamay, Sankaran, Wall, & Hennighausen, 1992).

The idea that S/MARs could insulate from methylation effects that cause gene silencing emerged from immunoglobulin (Ig) gene studies. In most pre-B cells, the κ chain gene is methylated and transcriptionally inert whereas in B- and plasma-cells the κ chain is hypomethylated and transcriptionally active. Studies showed that B-cells transfected with Ig κ transgenes were only demethylated when S/MAR was present (Lichtenstein, Keini, Cedar, & Bergman, 1994). Indeed, other studies found that the S/MARs are able to initiate transcription from CpG methylated genes (Forrester, Fernandez, & Grosschedl, 1999). Furthermore, S/MAR binds to chromatin remodeling proteins such as Bright, SAF-A, and p300, which in turn affect histone acetylation and remodeling of nucleosomes (Girod & Mermod, 2003).

Activation of transcription is accompanied by the anchoring of S/MARs to the nuclear matrix. This results in the formation of anchored chromatin loops that are insulated from the stimulatory or repressive effects of flanking chromatin. The transcription machinery is assembled at the site of S/MAR and brings together gene-coding sequences and regulatory elements, thus enabling coordinated regulation of specific genes. At the end of the S-phase, the replication machinery is dismantled (Fiorini et al., 2006).

12. TRANSCRIPTION AUGMENTATION

S/MARs may also act in *cis* to increase transcription initiation rates, even in the absence of an enhancer. However, how these S/MAR switch on gene expression remains unclear. One suggestion is that the single chromatin loops formed by the attachment of S/MAR to chromatin is not immobile and switching on gene expression involves changing the attachment points of the loops to the nuclear matrix (Kalos & Fournier, 1995). This suggests that the S/MAR is able to regulate gene expression by mediating changes in the structure of chromatin increasing the likelihood of establishing an active locus (Ottaviani, Lever, Takousis, & Sheer, 2008). Harraghy et al. reported that the inclusion of an S/MAR increases the probability of acquiring a permissive state while decreasing the occurrence of

silencing events associated with transgene integration in chromosomes of mammalian cells (Harraghy, Gaussin, & Mermod, 2008).

In addition to their chromatin-remodeling activities, S/MAR-binding transcription factors may also contribute to transgene regulation by directly interacting with components of the general transcription machinery. S/MAR binds to ubiquitous nuclear matrix proteins such as SAF-B and SATB1, which in turn associates with RNA polymerases, thereby increasing the accessibility of the pDNA to a "transcription factory." Consistent with this are FISH analyses showing pDNA pEPI clusters in active sites located at the periphery of the chromosome, where the chromatin remains open with easy access to transcription complexes (Allen et al., 1996; Mielke, Kohwi, Kohwi-Shigematsu, & Bode, 1990) (See Figure 2).

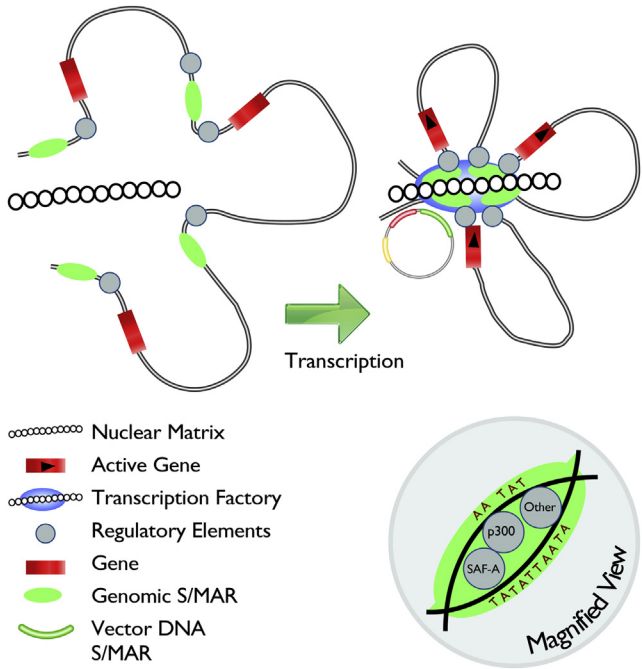

Figure 2 ***Schematic model depicting function of S/MAR in gene regulation.*** The activation of transcription is accompanied by the anchoring of S/MAR motifs to the nuclear matrix. This results in the formation of anchored chromatin loops that are insulated from the stimulatory or repressive effects of flanking chromatin. The transcription machinery is assembled at the site of S/MAR and brings together gene-coding sequences and regulatory elements, thus enabling coordinated regulation of specific genes. At the end of the S-phase, the replication machinery is dismantled. Magnified view illustrates the interaction of the AT-rich S/MAR motifs to components of the nuclear scaffold (Fiorini et al., 2006). (For interpretation of the references to color in this figure see the color plate.)

13. MITOTIC STABILITY

The molecular mechanisms behind the mitotic stability of pEPI *in vitro* remain unclear, but Stehle et al. showed that once the episome is established, it is efficiently maintained in active chromatin and associates with early replicating chromosomal sequences (Stehle et al., 2007). In contrast, vectors lacking an S/MAR or in which transcription terminates upstream of the S/MAR are liable to be integrated. Following integration, the loss of gene expression correlates with epigenetic silencing by promoter methylation (Jenke, Scinteie, Stehle, & Lipps, 2004). Jenke et al. suggested how the pDNA is segregated to daughter cells by showing that S/MAR binds to nuclear matrix proteins such as topoisomerase II, lamin B1, SATB1, and histone H1. In particular, S/MAR was found to associate preferentially with to SAF-A, a principal component of cellular chromatin and chromosomes. This suggests that during mitosis, pEPI interacts with mitotic chromosomes, mediated by the binding of S/MAR to SAF-A, enabling co-segregation with the chromosomes during mitotic division (Jenke et al., 2002). Furthermore, it is suggested that S/MAR recruits nuclear components in a manner similar to viral proteins, like Tag and EBNA-1, that lead to helix destabilization forming an open chromatin domain at the origin of replication necessary for assembly of the replicating machinery such as the ORC (see Figure 2).

14. PERSISTENT EXPRESSION MEDIATED BY S/MAR DNA VECTORS

The long-term expression mediated from an S/MAR pDNA vector was demonstrated in hematopoietic stem cells in 2006 (Papapetrou et al., 2006) where the vector was shown to remain episomal for a period of at least four weeks in the human hematopoietic progenitor cell line K562, murine erythroleukemia (MEL) cells, and in CD34+ cell lines after transfection. Interestingly, the pDNA vector was maintained as an episome even after it was silenced in the MEL cell line by histone deacetylation. Other mammalian cell lines used where S/MAR pDNA vectors have been shown to be retained episomally include CHO (Piechaczek et al., 1999), HeLa, human hepatoma (Huh7), human lung epithelial IB3 (our own observations), and in pluripotent p19 embryonic carcinoma cells (Sotirova et al., 2006). S/MAR pDNA vectors have also been used to express a short-hairpin RNA against the *bcr-abl* gene, in the K562 cell line where they were

demonstrated to exist as an episome for a period up to 4 months without drug selection pressure (Jenke et al., 2005). Importantly, they were demonstrated to remain functional over this time and were shown to suppress hepatitis B viral replication by approximately 77% over a period of eight months (Jenke et al., 2008). S/MAR DNA vectors have also been used to generate an HSV-1 amplicon vector that was shown to remain episomal for at least 11 weeks and enabled the complete restoration of human LDLR function in CHO $ldlr^{-/-}$ a7 cells to physiological levels (Lufino et al., 2007) throughout this time. Significantly, the S/MAR vector was used to create genetically modified pigs using the sperm-mediated gene transfer method. In 12 of 18 fetuses the vector was found in an episomal status at a number <10 copies/cell and reporter gene expression was confirmed in nine of the 12 episome-positive fetuses (Manzini et al., 2006).

However, despite the success of the original pEPI cytomegalovirus (CMV)-driven S/MAR vector *in vitro*, it was unable to provide sustained gene expression in mice following delivery to the liver or lung (Argyros et al., 2008; Conese, Auriche, & Ascenzioni, 2004). However, when the CMV promoter of pEPI was replaced by the liver-specific α1 antitrypsin (AAT) promoter, transgene expression was shown to persist in the mouse liver for at least six months (Argyros et al., 2008) and during continued evaluation was sustained for the duration of the animal's lifetime from a single administration (our own observations). In comparison the pDNA vector without the S/MAR element was silenced within the first week following its administration. More recently, a study showed strong sustained transgene expression following intrathecal delivery of an S/MAR-based vector in the murine CNS (Hughes et al., 2009).

15. ALTERNATIVE ELEMENTS TO MODULATE TRANSCRIPTION LEVELS OF DNA VECTORS

In addition to S/MAR, numerous studies have described other elements capable of shielding the transgene from positional effects and preventing interaction of the transgene with regulations of genes in its vicinity. To date, at least three elements other than S/MAR have been described: insulators, locus control regions (LCR), and ubiquitous chromatin opening elements (UCOEs).

Insulators are DNA sequences with the specific ability to protect from position effects. They are able to partition the genome into separate functional domains, so these can be independently regulated without disruption from

overlapping signals between promoters and enhancers at different loci. This manifests as an ability to block the action of an enhancer upon a promoter when placed between the two (Kellum & Schedl, 1992). The incorporation of an insulator present in the chicken β-locus in adenoviral (Steinwaerder & Lieber, 2000) and AAV vectors (Fitzsimons, McKenzie, & During, 2001) shields transgenes from the effects of flanking viral sequences and in integrated pDNA vectors increases the probability of achieving reporter gene expression (Johansen, Tornoe, Moller, & Johansen, 2003).

LCRs are a class of strong, long-range transcriptional enhancers that possess dominant chromatin-opening ability, and are therefore able to provide copy number dependent expression to transgenes to which they are linked. The activity of most LCRs is tissue specific, however, a small number of UCOEs have been identified in the regions surrounding the promoters of certain housekeeping genes (Antoniou et al., 2003). The inclusion of a hepatocyte-specific LCR derived from the apoliprotein E (apoE) locus in pDNA showed enhancement of human factor VIII and IX transgenes in a liver-specific manner (Miao, Ye, & Thompson, 2003).

While these elements provide singular functions, S/MARs are versatile and provide a combination of these properties. Similar to insulators, S/MARs prevent the spread of heterochromatin and negative environment effects upon inclusion in a plasmid. The difference between insulators and S/MARs is that insulators mark the boundaries of active domains within a chromosome while S/MARs are the boundaries by which chromatin is physically constrained into loops. This physical linkage to the nuclear scaffold provides mitotic stability and segregation to daughter cells. In addition S/MARs, like LCRs and UCOEs, are able to mediate domain opening facilitating transcription by maximizing the probability of interaction between promoters and other transcription factors with the transgene. Recently, Hagedorn et al. showed a synergy between these elements and showed that the incorporation of a UCOE enhanced the expression of established S/MAR DNA vectors while the addition of an insulator sequence (cHS4) increased the efficiency of establishment, presumably via an additional interaction with the nuclear matrix (Hagedorn, Antoniou, & Lipps, 2013).

16. OPTIMIZATION OF EPISOMAL VECTORS FOR GENE THERAPY

The degree of therapeutic correction achieved is dictated by the success of gene transfer. Once genetic material is introduced into a target cell,

sufficient protein expression is required for realization of gene therapy. There are two approaches to increase the amount of protein levels for therapeutic benefit, by increasing the number of cells transduced or by increasing the amount of protein produced by each transduced cell. Since nonviral vectors have a limited transduction efficiency, for example, 30–40% of total hepatocytes following hydrodynamic delivery, maximizing the output of therapeutic protein for successfully delivered expression cassettes is necessary. The majority of gene therapy vectors currently in use contain a simple, generic expression cassette under the control of a promoter. In addition to S/MAR incorporation, the simplest approach to alter expression characteristics of a transgene cassette is to include promoters and enhancers that can drive strongest possible expression specific to the target cell. In addition, improved levels of gene expression can be achieved by the elimination of unnecessary CpG motifs, which may reduce immune reactions and post-transcriptional modifications to enhance the processing of mRNA.

17. PROMOTERS AND ENHANCERS

The promoter is perhaps the key regulatory element that determines the strength of the transgene expression from a pDNA vector. Within the promoter are binding sites for RNA polymerase and their associated factors that initiate transcription. An enhancer is a separate DNA sequence, which binds proteins other than RNA polymerase, such as transcription factors and chromatin-remodeling complexes, and acts upon promoters to enhance their transcription efficiency. Depending on the presence of associated upstream or downstream enhancer elements, the promoter can range from 100 to 2000 bp in length.

When contained in a pDNA, the promoter is not subject to the same controls as endogenous genes, making it difficult to predict the performance of the promoter simply by sequences. Both promoters and enhancers can be ubiquitously active or stringently tissue specific, depending on the expression profile of the binding factors required for their activity and the variety of both types of elements in vectors. The same promoter and enhancer that confer long-term expression in a viral vector may provide only transient expression in a pDNA. Many promoters are only able to confer short-term expression when contained in nonviral vectors, most likely due to loss of vector DNA from transduced tissue or transcriptional inactivation of the promoter. The incorporation of an S/MAR motif in a DNA vector

has, however, been shown to prevent the silencing of tissue-specific promoters in an episomal DNA vector (Argyros et al., 2008) perhaps by restoring a natural cellular environment for their expression.

In addition to localizing transcription targeting, using specific promoters to drive transgene expression could also circumvent the immune responses. For example, the expression of AAT by its natural AAT promoter was over a 100-fold higher compared to expression driven by the ubiquitous PGK or CMV promoter (Schiedner et al., 2002) and gene expression from a muscle-specific promoter is superior over the immunogenic, transiently expressed CMV promoter in the muscle (Cordier et al., 2001).

18. THE CMV PROMOTER

The promoter from the immediate early gene of human CMV is one of the most widely used promoters as it confers robust expression in most cell types tested (Guo, Wang, Eisensmith, & Woo, 1996). This strong activity is due to the presence of multiple repeats of several transcription binding sites within the promoter and upstream enhancer region (Boshart et al., 1985). The expression profile of this promoter typically peaks 1–2 days following administration of the vector. The reasons behind the rapid inactivation are uncertain, but are suggested to be due to several reasons, such as cytokine inhibition (Zhang et al., 1995) and activation by repressor proteins that cause methylation (Sinclair, Baillie, Bryant, Taylor-Wiedeman, & Sissons, 1992; Zhang et al., 1995).

Promoters from genes that encode abundant cellular proteins, such as β-actin and elongation factor 1α (EF1α), have been applied with some success in providing sustained transgene expression, although their expression is considered to be lower than that conferred by the CMV promoter. For example, the EF1α promoter, from the gene encoding EF1α which is an abundant, widely expressed protein responsible for catalyzing GTP-dependent binding of tRNA to ribosomes, resulted in a 10-fold lower marker gene expression compared to that conferred by CMV but declined much more gradually unlike CMV and was still detectable four weeks post-instillation in the lung (Gill et al., 2001).

19. THE UbC PROMOTER

More promising is the UbC promoter, which drives expression from three known cellular human ubiquitin genes, UbA, UbB, and UbC.

Ubiquitin is abundantly expressed in all eukaryotic cells and attaches covalently to abnormal, misfolded, or short-lived proteins marking them for destruction. The UbC promoter is shown to provide high-level, ubiquitous expression when inserted into transgenic mice (Schorpp et al., 1996). When included in pDNA vectors, equally strong levels of reporter gene expression was detected in the lung for two months after instillation, but gradually declined and were lost six months after administration (Gill et al., 2001). A hybrid of the CMV-UbC promoter was able to provide sustained expression for up to 84 days after lung instillation and for 42 days following hydrodynamic delivery to the liver (Yew, Przybylska, Ziegler, Liu, & Cheng, 2001).

20. THE AAT PROMOTER

The human AAT gene is one of the major proteinase inhibitors in the serum and is synthesized mainly in hepatocytes although it is also expressed by macrophages during inflammation. Hepatocytes and macrophages transcribe the same coding region of the AAT gene from promoters about 2 kb apart, but only the 305 bp hepatocyte-specific promoter is able to drive expression in liver cells. An investigation of this hepatocyte control region revealed that it is able to drive full hepatocyte-specific expression. The AAT promoter was able to sustain therapeutic levels of factor VIII and IX for up to six months after hydrodynamic delivery (Miao et al., 2003) and reach the highest levels of AAT expression to date after hydrodynamic gene transfer (Alino, Crespo, & Dasi, 2003). The liver is an immune privileged organ (Knolle & Gerken, 2000) and the employment of liver-specific promoters for targeted hepatocyte expression has a distinct advantage over the use of more widely expressed promoters such as CMV or UbC, in terms of avoiding potential immune responses from the expressed protein. Restricting expression to hepatocytes is reported to reduce the antibody response to a foreign protein significantly (Pastore et al., 1999), as evading the immune system is an important factor in achieving long-term expression *in vivo*.

21. ENHANCERS

As another technique to produce better expression cassettes, different promoter–enhancer combinations have been considered for incorporation

into gene therapy vectors. Most native promoters contain one or two enhancer elements fused to a heterologous promoter sequence. To produce higher promoter activity, studies have investigated the possibility of combining the enhancer elements endogenously present in control regions of genes to promoters and the effect of enhancer/promoter combinations on transgene expression. A recent study has identified hepatic transcription factor-binding site motifs, called "regulons," which are associated with highly expressed liver genes and are evolutionary conserved among divergent species. The combination of different regulons with basic hepatocyte-specific promoters resulted in a 20- to 40-fold increase in FXI expression following hydrodynamic delivery *in vivo* (Petrus et al., 2008), although this is relatively newly discovered and still being tested.

Another liver-specific enhancer was revealed in the human apolipoprotein (*apo*) gene cluster, which expresses apoE, C-I, and C-II proteins. ApoE is expressed predominantly in the liver, but to some extent in brain, skin, spleen, and kidney and mediates clearance of low-density lipoproteins and excess cholesterol from peripheral tissues. The *apo* gene cluster contains two hepatic control regions (HCRs), HCR-1 and HCR-2. Within HCR-1, a 154-bp fragment containing a liver-specific enhancer was shown to act as a general hepatocyte-specific enhancer and was able to direct full liver *apoE* expression in transgenic mice (Shachter, Zhu, Walsh, Breslow, & Smith, 1993). De Geest et al. combined one, two, and four copies of the *apoE* enhancer with a liver-specific promoter and demonstrated significantly higher and sustained transgene expression driven by the four copies of enhancer/promoter combination over that of the promoter alone (De Geest, Van Linthout, & Collen, 2001; De Geest, Van Linthout, Lox, Collen, & Holvoet, 2000). When four copies of the *apoE* enhancer are combined with the AAT promoter (together called the A4 promoter), a higher level of transcription was induced over that of conventional liver promoters (Lam et al., 2007).

22. POSTTRANSCRIPTIONAL REGULATION

While selection of a strong promoter is the first step to ensure high-level transcription of mRNA, the posttranscriptional events concerning mRNA processing can also be enhanced, either by increasing stability of this message after transcription, promoting its export from the nucleus, or increasing the efficiency of translation. One approach is the inclusion of an intron sequence at the 5′ or 3′ end of the RNA of interest. A majority

of naturally occurring eukaryotic genes are interrupted by introns and expression from these intron-containing transcripts are often higher than from the equivalent intron-deficient cDNA sequences (Cullen, 2003). In some cases, expression is entirely dependent on the presence of an intron, for example, β-globin (Buchman & Berg, 1988). Although the exact mechanism is unclear, it is suggested that the intron, or the process of mRNA splicing itself, may promote 3′-end formation, enhance mRNA stability in the nucleus, and/or improve RNA export to the cytoplasm (Brun, Faucon-Biguet, & Mallet, 2003), which in turn efficiently routes messages to the ribosome for translation (Clouse, Luo, Zhou, & Reed, 2001).

Another element capable of stimulating gene expression posttranscriptionally is called a posttranscriptional regulatory element (PRE). The hepatitis B virus (HBV) encodes several intronless mRNA and employs a *cis*-acting PRE RNA element for cytoplasmic accumulation of viral RNAs (Huang & Liang, 1993). This element called the HPRE contains two stem loops that confer its function. It compensates for the absence of introns by directing the viral transcripts into a Crm1-dependent cellular nuclear export pathway. The related and another hepadnavirus, woodchuck hepatitis virus (WHV), possesses a similar element called the WPRE. In addition to the double-stem loop in the HPRE, the WPRE contains a third functional domain (the γ element), which provides an increased enhancement of transgene levels, above that conferred by the HPRE (Donello, Loeb, & Hope, 1998). The WPRE has widely been used as a *cis*-acting regulatory module in various types of pDNA or viral gene vectors to increase transgene expression from gene therapy vectors. Although the WPRE is coupled in part to the Crm1-dependent export machinery, its posttranscriptional enhancement does not result from increased RNA export or from an increased rate of transcription but rather from the improved 3′ transcript processing. When placed in the 3′-untranslated region of expression cassettes, it appears to stimulate 3′ end polyadenylation in the nucleus thereby preventing tail degradation in the cytoplasm (Higashimoto et al., 2007). The WPRE appears to increase the amount of polyadenylated transcripts and clearly augments the size of the polyA tail of RNA (Schambach, Galla, Maetzig, Loew, & Baum, 2007) and its action is independent of the vector, promoter, and transgene used and of the proliferative status of the transduced cells (Brun et al., 2003). There have been, however, some safety concerns regarding the inclusion of the WPRE in gene therapy vectors. This is due to overlap of its sequence with that of the WHX protein, which is a transcriptional activator implicated in the development of liver tumors.

The WPRE inserted in most vectors contains the WHX gene promoter and an open reading frame coding for the first 60 amino acids of WHX in its 3′ region. This polypeptide represents about 80% of the C-terminally truncated sequences of the X protein gene that were retrieved from liver tumors containing the integrated WHV or HBV genome (Wei, Etiemble, Fourel, Vitvitski-Trepo, & Buendia, 1995). The C-terminal portion of the X protein appears to be important in the regulation of cell proliferation and viability. However, there is no evidence that such polypeptide could be expressed or functional when inserted in a heterologous transcription unit like a vector. C-terminal truncated mutants are suggested to cooperate with other tumor suppressor genes increasing susceptibility to carcinoma (Kingsman, Mitrophanous, & Olsen, 2005). These indirect arguments suggest that WPRE sequences may contribute to tumor progression but is insufficient alone to drive tumor progression. Subsequent experiments, however, showed that mice treated with lentiviruses bearing the WPRE or a WPRE mutated at the X gene promoter did not develop tumors (Themis et al., 2005). Indeed, no experimental evidence of cellular transformation has been demonstrated following the administration of expression cassettes containing a WPRE element.

23. EFFECT OF CpG DEPLETION

As mentioned earlier, the innate immune system is capable of identifying pDNA principally by its higher frequency of unmethylated CpGs compared to mammalian DNA (Krieg, 2000). Chen et al. showed that both the persistence and silencing of transgene expression were associated with specific increases in heterochromatin-associated histone modifications and a subsequent decrease in modifications associated with euchromatin (Chen, Riu, He, Xu, & Kay, 2008). Unmethylated CpG motifs are a major contributor to the acute inflammatory response that occurs following administration of cationic-lipid pDNA complexes (Yew et al., 1999). Modifying nonviral vectors by reducing their unmethylated CpG content, e.g., by methylating or removing CpGs from the pDNA, can avoid immune responses. It has been reported that certain sequence motifs specifically inhibit the recognition and signal transduction of CpG oligo deoxynucleotides (for example, replacing a GCGTT or ACGTT motif with GCGGG or ACGGG, respectively) and converted a stimulatory CpG oligo deoxynucleotide to an inhibitory one preventing apoptosis induced by stimulatory

CpG oligo deoxynucleotides (Rothenfusser, Tuma, Wagner, Endres, & Hartmann, 2003). Deletion of the CpG content in a pDNA after successive rounds of site-specific mutagenesis has been shown to result in a higher persistent level of transgene expression in mice (Yew et al., 2002). However, methylation or mutation of CpGs may affect activity of the promoter or reduce the pDNA's replication capabilities, as CpGs in pDNA vectors are usually close to the promoter or *ori*. For example, reduction of CpGs from the CMV promoter has been found to reduce activity by <50% (Yew et al., 2002).

An alternative method to reduce unmethylated CpG content is to produce minimally sized DNA vectors comprising only the expression cassette (promoter-transgene-poly A signal) thereby removing any extraneous elements not required for transcription of the transgene of interest in eukaryotic cells. These constructs are called "minicircles" and contain neither the bacterial *ori* nor antibiotic resistance genes (the bacterial backbone). Minicircles were shown to confer strong long-term expression by retaining histone patterns consistent with euchromatin (Chen et al., 2008). In another study, the minicircles were maintained intact in episomal form in the hepatocytes *in vivo*, and mediate up to 560-fold increase in transgene expression over bacterial backbone-harboring pDNAs (Chen, He, Ehrhardt, & Kay, 2003). The superiority of minicircles over that of standard pDNA has been shown *in vivo* using reporter proteins (Vaysse et al., 2006), expression of human manganese superoxide dismutase that protect from radiation (Zhang et al., 2008), and other human proteins such as AAT (Chen et al., 2003) and vascular endothelial growth factors (Chang, Christensen, Lee, & Kim, 2008).

Several strategies have been developed to create minicircles, mainly utilizing site-specific recombination of integrase systems such as phiC31 (Chen et al., 2003), λ integrase (Darquet et al., 1999), Flp recombinase (Nehlsen, Broll, & Bode, 2006), or Cre recombinase (Bigger et al., 2001) (See Figure 3). As an illustration for the production of minicircles using Cre recombination, plasmids (called producer plasmids) are prepared containing two 34-bp LoxP sites, which flank the pDNA's expression cassette. Under tight control of an arabinose expression system, intramolecular recombination is mediated by Cre recombinase between the LoxP sites when the LoxP are in the correct orientation. This site-specific recombination produces two supercoiled DNA molecules that are unlinked, each containing a single LoxP site. The circle consisting of the expression cassette is called the minicircle, whereas the circle containing the bacterial backbone is called

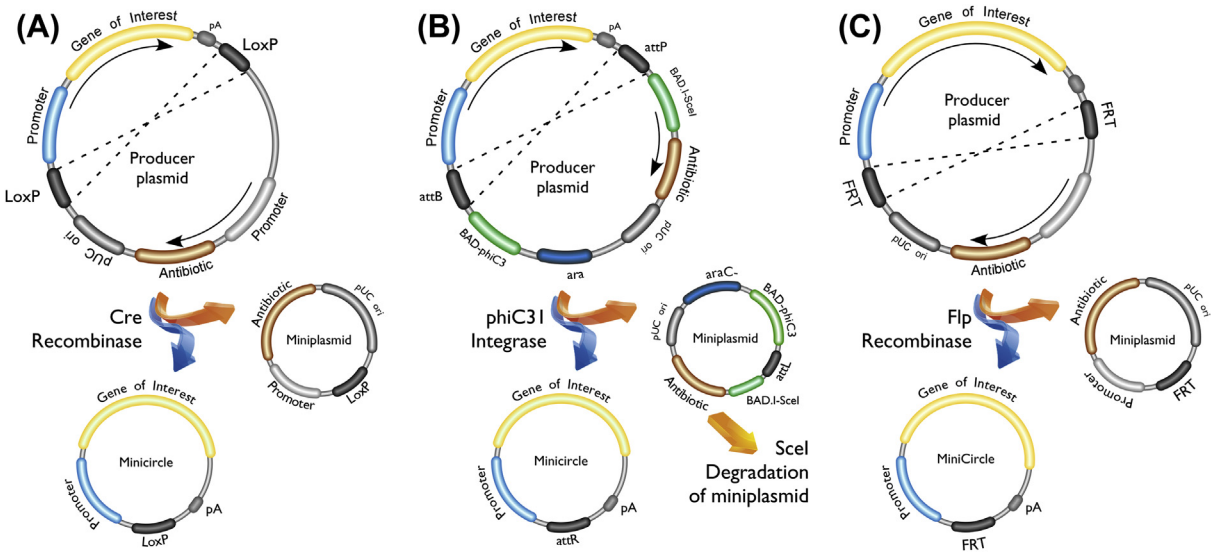

Figure 3 ***Schematic representation of the three most commonly used methods for minicircle production.*** (A) Generation of minicircles using the Cre/Lox recombination method, where Cre recombinase catalyzes specific recombination of DNA between loxP sites. (B) Generation of minicircles using the C31 integrase, which catalyzes the site-specific recombination of two recognition sites that differ in sequence, typically known as attachment sites attB and attP. Typically, miniplasmids are subsequently digested *in vitro* by SceI. (C) Generation of minicircles using the Flp recombinase, which catalyzes recombination between the 34-bp-long flippase recognition target (FRT) sites. Table 1 summarizes the application of vectors produced using these systems. (For interpretation of the references to color in this figure see the color plate.)

miniplasmid. Since Cre recombination is bidirectional, LoxP sites were modified at the terminal 5′ nucleotide to create a left element LoxP site and vice versa to create a right element LoxP site in order to shift the equilibrium toward the production of minicircles. After recombination, the minicircle contains a hybrid LoxP site with impaired recombination ability while the bacterial backbone miniplasmid contains a wild-type LoxP site. This strategy efficiently directs the drive toward minicircle production and reduces minicircle concatamerization due to Cre's reduced affinity for the hybrid LoxP site (Bigger et al., 2001).

Several groups have generated minicircle vectors expressing a variety of different transgenes in a range of cells or tissues (Table 1). In almost every case, these minimally sized vectors showed improved and prolonged levels of gene expression *in vitro* and *in vivo*.

24. STUDIES DEMONSTRATING PASSIVE EPISOMAL STATE OF MINICIRCLES *IN VIVO*

Chen et al. reported the successful production of minicircles using the φC31 recombinase in 2003 (Chen et al., 2003). These minicircles encoded either the human AAT cDNA from the RSV promoter, or the human FIX gene from an artificial enhancer/promoter (sAPOE) and were delivered in the mouse hydrodynamically and compared to equimolar amounts of pDNA vectors bearing the same expression cassettes.

Results showed that mice administered with minicircle DNA produced up to 500-fold higher expression than that of the group dosed with pDNA. Mice receiving circular pDNA also expressed a high level of serum human AAT initially, but the serum reporter level dropped precipitously over 700-fold during the first three weeks and continued to decline. The data clearly demonstrated that the minicircle was an efficient vector form and could provide persistent expression and relatively high levels of transgene product compared to the originating pDNA vector.

The same group also compared the human FIX-expressing minicircle to the corresponding pDNA. Animals dosed with minicircle produced significantly higher and stable levels of serum human FIX for up to seven weeks following a single administration. In contrast, although the animals injected with pDNA showed initially higher levels of human FIX 24 h after pDNA delivery, transgene expression dropped more nearly 50-fold within three weeks and steadily declined afterward.

Table 1 Summary of studies performed *in vitro* and *in vivo* using minicircle vectors

Promoter utilized	Transgene	Integrase or recombinase	Cell line	Tissue specificity	Episomal status: *in vitro*/*in vivo*	Mode of administration	Reference
Human UbC, human AAT	Luciferase	Cre	U251	Mouse liver	Yes/yes	Hydrodynamic injection	Argyros et al. (2011)
CMV, SV40	eGFP	Flp	HEK293, CHO-K1, NIH 3T3, mouse and bovine zygote	—	Yes/—	—	Broll, Oumard, Hahn, Schambach, and Bode (2010), Nehlsen et al. (2006)
CMV	Human MnSOD cDNA	φC31	32D cl 32C6	Esophagus	?/?	Lipoplexes in mouse esophagus or through systemic tail vein injection	Zhang et al. (2008)
SV40, CMV, or chicken β-actin	Human VEGF cDNA	φC31		Mouse skeletal muscle	?/?	Intramuscular injection of naked DNA	Chang et al. (2008)
Hybrid sAPOE and human EF1a-CMV, SV40, human heIF, and human GAPDH	Human FIX cDNA-human α1-antitrypsin	φC31	—	Mouse liver	—/yes	Hydrodynamic injection	Chen, He, Meuse, and Kay (2004)

(*Continued*)

Table 1 Summary of studies performed *in vitro* and *in vivo* using minicircle vectors—cont'd

Promoter utilized	Transgene	Integrase or recombinase	Cell line	Tissue specificity	Episomal status: *in vitro*/*in vivo*	Mode of administration	Reference
CMV	Human VEGF cDNA	φC31	HT-1080	Mouse heart and skeletal muscle	?/?	Intramyocardial or intramuscular injection of naked DNA	Stenler et al. (2009)
Human UbC	Human HIF-1A cDNA	φC31	C2C12	Mouse heart	?/?	Intramyocardial injection of naked DNA	Huang et al. (2009)
CMV	Oct4, LIN28, NANOG, SOX2 cDNAs, and eGFP	φC31	Human adipose stem cells	—	?/—	—	Zhang et al. (2010)
CMV	Human IFNγ cDNA	φC31	HEK293, NIH 3T3, CNE-1, CNE-2, C666-1	Human nasopharyngeal carcinoma	?/?	Lipoplex	Wu et al. (2006)
Chicken β-actin	Human VEGF cDNA	φC31	HEK293, NIH 3T3, CHO-K1	Mouse skin	?/?	Sonoporation with microbubbles	Yoon et al. (2009)

SV40	Luciferase	λ integrase	NIH 3T3, 3LL, H460, RSM, HSM	–	?/–	–	Darquet et al. (1999)
CMV	β-Galactosidase	Cre	A549, NIH 3T3	Mouse liver and lungs	?/?	Tail vein injection of liposomes followed by naked DNA	Vaysse et al. (2006)
CMV	eGFP and luciferase	φC31	SKOV3, H22, HeLa, A431, HepG2,	Mouse tumor xenograft	?/?	Tail vein injection of folate-polyplex	Zhang et al. (2010)
DC190, AAT DC172, LSP	Human apoAI human FIX	φC31	–	Mouse liver	–/?	Hydrodynamic injection	Jacobs et al. (2008)
Human AAT	Human FIX	φC31	–	Mouse liver	–/?	Hydrodynamic injection	Schuttrumpf et al. (2009)
Hybrid sAPOE	Human IDUA	φC31	–	Mouse liver	–/yes	Hydrodynamic injection	Osborn et al. (2011)
Synthetic regulatory element, P3	Murine PAH	φC31	–	Mouse liver	–/yes	Hydrodynamic injection	Viecelli et al. (2014)

The episomal status of the administered vectors was subsequently analyzed by Southern blot analysis at 15 weeks postdelivery (Chen et al., 2003) which showed that both the minicircle DNA as well as the pDNA had not integrated and remained episomally within the hepatic nuclei.

In subsequent experiments, minicircle vectors were evaluated driven by a variety of promoters such as UbC, CMV, SV40, heI4AF1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter, and, in every case, they showed more persistent and higher levels of transgene expression compared to the originating pDNA. These experiments clearly showed the deleterious and inhibitory effect of the bacterial DNA in a eukaryotic cell and that this was transgene and promoter/enhancer independent (Benham & Bi, 2004; Riu, Chen, Xu, He, & Kay, 2007). More recently, Maniar et al. have demonstrated that these active minicircle vectors have different methylation and patterns of chromatin insulation compared to pDNA and that this leads to protection against transcriptional silencing (Gracey Maniar et al., 2013).

Another study directly compared pDNA with minicircle DNA using several different hepatocyte-specific promoters in mice following hydrodynamic delivery. In every case although the expression of minicircle DNA was superior to the pDNA, the DNA from each group was lost from hepatocytes at a similar rate probably due to the turnover of hepatocytes in the liver. This was illustrated more clearly in experiments following the fate of minicircle DNA in the liver after partial hepatectomy on mice that had been injected with minicircle DNA or pDNA (Argyros et al., 2011; Jacobs et al., 2008), with both forms of vector rapidly lost during rapid cell division in the regenerating liver.

Finally, recent papers have described the use of minicircle vectors for the genetic correction of mouse models of disease. Osborn et al. described the application of minicircles to a model of Hurler Syndrome, a lysosomal storage disease, (Osborn et al., 2011) and Viacelli et al. described their application to a mouse model of phenylketonuria, a monogenic metabolic liver defect (Viecelli et al., 2014). In both studies, the minicircle vector showed prolonged and corrective gene expression compared to the pDNA controls and Southern blot analysis showed the episomal status of the vector in the mouse liver.

25. CONCLUSIONS

Great progress has been made over the past decade in deciphering and understanding some of the molecular mechanisms, which influence the level

and duration of expression of transgenic material and its maintenance within a cell. Novel, exquisitely designed expression vectors are being generated and applied which incorporate more efficient promoters along with genetic components that can enhance the expression of transgenic material while preventing its silencing as well as facilitating its sustenance and even replication. Additionally, technologies have been developed to improve the preparation of the recombinant DNA vector itself with the removal of extraneous and potentially damaging sequences that are not required for function in mammalian cells. These minimally sized DNA vectors show great promise providing improved levels and duration of transgene expression. Their utility has recently been demonstrated to great effect with long-term genetic correction in preclinical models of human disease. DNA vectors therefore remain a viable alternative system to recombinant viruses in clinical application.

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CHAPTER SIX

Noncoding Oligonucleotides: The Belle of the Ball in Gene Therapy

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Contents

Abstract

Gene therapy carries the promise of cures for many diseases based on manipulating the expression of a person's genes toward the therapeutic goal. The relevance of noncoding oligonucleotides to human disease is attracting widespread attention. Noncoding oligonucleotides are not only involved in gene regulation, but can also be modified into therapeutic tools. There are many strategies that leverage noncoding oligonucleotides for gene therapy, including small interfering RNAs, antisense oligonucleotides, aptamers, ribozymes, decoys, and bacteriophage phi 29 RNAs. In this chapter, we will provide a broad, comprehensive overview of gene therapies that use noncoding oligonucleotides for disease treatment. The mechanism and development of each therapeutic will be described, with a particular focus on its clinical development. Finally, we will discuss the challenges associated with developing nucleic acid therapeutics and the prospects for future success.

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1. INTRODUCTION

Gene therapy promises a future where genetic diseases, such as Alzheimer's disease, cystic fibrosis, Parkinson's disease, and inherited blindness, are no longer chronically managed, but instead mitigated with a single robust intervention. The revolutionary idea of gene therapy is to correct the faulty gene by introducing manipulated genetic material into the human genome. Since the early 1990s, more than 1900 clinical trials have been conducted with various gene therapy approaches (Kaufmann, Buning, Galy, Schambach, & Grez, 2013). The initial growth trajectory of gene therapy trials was slow because of the tragic death of a young man who suffered from a lifetime mild deficiency of ornithine transcarbamylase and experienced complications from the gene therapy (Stolberg, 1999). Safety and efficacy concerns diminished optimism for the projected clinical and commercial potential of gene therapy products. Despite these initial setbacks, basic research projects kept churning along in an effort to reduce the side effects of gene therapy. This research led to the approval of the gene therapy product Glybera® (Alipogene tiparvovec) for the treatment of lipoprotein lipase deficiency in Europe (Yla-Herttuala, 2012) and an adenovirus-p53-based therapy, Gendicine® (rAd-p53), for the treatment of head and neck squamous cell carcinoma in China (Wilson, 2005). There is no approved gene therapy product in the United States yet, but excitement and investment in gene therapy have been immensely revived.

Noncoding oligonucleotides, a strand of nucleic acids with less than 100 base pairs (bp), are the most well-studied entities in gene therapy. In contrast to typical protein-coding RNA molecules, noncoding oligonucleotides do not store genetic information, but play diverse and significant roles in complex biological systems. In humans, evolutionarily conserved noncoding oligonucleotides account for 98% of the genome and are divided into several categories according to their size and function (Kapranov et al., 2007; Ma, Bajic, & Zhang, 2013). These noncoding oligonucleotides, such as microRNAs (miRNAs), small nucleolar RNAs, large intergenic ncRNAs, transcribed ultraconserved regions, and PIWI-interacting RNAs, are transcribed using the cellular machinery in a developmentally regulated and tissue-specific fashion (Carninci et al., 2005). In addition, noncoding oligonucleotides are involved in genetic and epigenetic events in the context of cancer and other human disorders, such as cardiovascular, autoimmune, neurological, and developmental (Esteller, 2011; Harries, 2012).

Within the cell, noncoding nucleic acids primarily function in gene regulation, and their properties can be adapted for therapeutic applications. Recent transcriptional and bioinformatics studies have revealed the complexity and interplay among the noncoding nucleic acids (Tay, Rinn, & Pandolfi, 2014). A detailed survey of these regulatory noncoding oligonucleotides is beyond the scope of this book, but the reader is advised to consult recent reviews that address this topic thoroughly (Cech & Steitz, 2014; Fatica & Bozzoni, 2014; Geisler & Coller, 2013; Tay et al., 2014). In this chapter, we will discuss the use of noncoding oligonucleotides in gene therapy applications. Noncoding oligonucleotides can be artificially designed and adopted as new therapeutic modalities for the treatment of a gamut of diseases by various mechanisms (Kole, Krainer, & Altman, 2012). Given their propensity to form complementary bp, noncoding oligonucleotides can fold into elegant three-dimensional structures capable of molecular recognition and even catalysis. The rules governing three-dimensional structure formation appear to be dictated by the base sequence, similar to Anfinsen's protein folding theory (Biro, 2005). Since the first demonstration of the catalytic properties of RNA, pioneered by Thomas Cech and Sidney Altman over two decades ago (Guerrier-Takada, Gardiner, Marsh, Pace, & Altman, 1983; Kruger et al., 1982), many noncoding oligonucleotides have been discovered, leading to the notion that noncoding oligonucleotides hold promise as a new class of therapeutics. Some familiar oligonucleotide-based strategies, such as antisense oligonucleotides and aptamers, have already led to products currently on the market. In this chapter, we will focus on six types of oligonucleotide therapeutics that are actively used in the gene therapy setting and highlight several promising candidates currently in the clinical pipeline.

2. NONCODING OLIGONUCLEOTIDES-BASED THERAPEUTICS IN GENE THERAPY

2.1 RNA Interference (siRNA and shRNA)

RNA interference (RNAi) is a cellular process for gene silencing based on recognition and subsequent degradation of specific mRNA sequences. The process was initially observed in plants (Anandalakshmi et al., 1998), and was later demonstrated in the nematode worm by Andrew Fire and Craig Mello (Tabara et al., 1999). This groundbreaking discovery won Fire and Mello the Nobel Prize in physiology or medicine in 2006. The RNAi process can be triggered by small interfering RNAs (siRNAs) or short

hairpin RNAs (shRNAs). siRNAs are short double-stranded RNA molecules (20–25 bp) with two well-defined overhanging nucleotides at the 3′ end, and shRNAs are longer double-stranded RNA molecules (~70 bp) that contain a tight hairpin turn. These ncRNAs can be artificially designed and chemically synthesized with ease. Also, there are rules for the design that can optimize the silencing capacity, stability, and immunostimulatory properties of these ncRNA molecules (Jagla et al., 2005). Once the siRNA or shRNA molecules are localized to the cytoplasm of the cell via the various delivery vehicles discussed in this book, they are processed by the cellular RNAi machinery. Typically, exogenously injected long shRNAs are handled by the Dicer/TRBP complex to produce a ~21 bp double-stranded RNA duplex—exactly the same structure as the canonical siRNAs. Subsequently, the ~21 bp siRNA product assembles with the active multiprotein RNA-induced silencing complex (RISC), which unwinds the double-stranded siRNA through an ATP-dependent process, loads the siRNA strand that is complementary to the target mRNA (guide strand) into the RISC, and discards the other siRNA strand (passenger strand). The assembled RISC then dictates the degradation of the target mRNA and suppression of gene expression (Mello & Conte, 2004; Song, Smith, Hannon, & Joshua-Tor, 2004). Because the RISC complex protects the loaded siRNA from nuclease attack in the cell and can be recycled to cleave another complementary mRNA transcript, picomolar to subnanomolar amounts of siRNAs in a single dose would be sufficient to produce long-lasting gene silencing (Ameres et al., 2010).

Since RNAi harnesses a natural pathway that can turn off gene expression at the transcriptional level even before protein translation occurs, synthetic siRNA/shRNA-based therapeutics possess several advantages over small molecules and biologics, which mostly block the physiological functions of proteins or inhibit the catalytic functions of enzymes (Kim & Rossi, 2007). One major advantage is that siRNA/shRNA therapeutics can be created for any target, including targets that are considered "undruggable" (Bumcrot, Manoharan, Koteliansky, & Sah, 2006). Currently, there are approximately 22 different siRNA/shRNA therapeutics undergoing rigorous clinical testing for diseases ranging from genetic disorders to human immunodeficiency virus (HIV) infections and cancers (Burnett & Rossi, 2012; Burnett, Rossi, & Tiemann, 2011). Patisiran (ALN-TTR02), led by Alnylam® Pharmaceuticals, is currently in phase III clinical trials and represents the most advanced RNAi-based therapeutic (Table 1). Patisiran treats transthyretin (TTR)-mediated amyloidosis (ATTR) in patients with familial

Table 1 Examples of noncoding RNA drugs in the clinical pipeline

Drug name	Disease	Target	Status	Developer	Clinicaltrials.gov Identifier
siRNA/shRNA					
Patisiran (ALN-TTR02)	Familial amyloidotic polyneuropathy	*TTR*	Phase III, recruiting	Alnylam® Pharmaceuticals	NCT01960348
AGN-745 (SiRNA-027)	Wet age-related macular degeneration	*FLT1*	Phase II completed, terminated	Allergan® Inc.	NCT00395057
ALN-TTRsc	Familial amyloidotic cardiomyopathy	*TTR*	Phase I/II, recruiting	Alnylam® Pharmaceuticals	NCT01981837
Atu-027	Advanced solid tumors	*PKN3*	Phase I/II, recruiting	Silence Therapeutics®	NCT01808638
TKM-ApoB	Hypercholesterolemia	*APOB*	Phase I, terminated	Tekmira® Pharmaceuticals	NCT00927459
FANG™ Vaccine	Ovarian cancer, advanced melanoma, and colorectal cancer	*FURIN, GM-CSF/ CSF2*	Phase I/II, recruiting	Gradalis® Inc.	NCT01551745, NCT01309230, NCT01061840
DCR-MYC	Hepatocellular carcinoma	*MYC*	Phase I, recruiting	Dicerna Pharmaceuticals®	NCT02110563
Antisense					
Fomivirsen (ISIS 2922)	AIDS patients with CMV	CMV mRNA	Approved in 1998; discontinued in 2004	Isis Pharmaceuticals®	NCT00002187, NCT00002355
KYNAMRO® (mipomersen)	Homozygous familial hypercholesterolemia	*APOB-100*	Approved in 2013	Isis Pharmaceuticals®	NCT01475825, NCT00477594, NCT00607373

(*Continued*)

Table 1 Examples of noncoding RNA drugs in the clinical pipeline—cont'd

Drug name	Disease	Target	Status	Developer	Clinicaltrials.gov Identifier
ISIS-TTR_{RX}	Familial amyloid cardiomyopathy	*TTR*	Phase III, recruiting	Isis Pharmaceuticals®	NCT01737398
Eteplirsen (AVI-4658)	Duchenne muscular dystrophy (DMD)	(*DMD* exon 51)	Phase II	Sarepta Therapeutics®	NCT01396239, NCT01540409
Drisapersen	Duchenne muscular dystrophy (DMD)	(*DMD* exon 51)	Phase III	Prosensa Therapeutics®	NCT01803412
Miravirsen	Liver transplant (HCV)	miR-122	Phase II	Santaris Pharma A/S®	NCT01200420, NCT01872936
AVI-7100	Influenza	Influenza mRNA	Phase I, recruiting	Sarepta Therapeutics®	NCT01747148 (Sponsored by National Institute of Allergy and Infectious Diseases)
RG-101	HCV infection	miR-122	Phase I	Regulus Therapeutics®	N.A.
Aptamer					
MACUGEN®	Age-related macular degeneration	$VEGF_{165}$	Approved in 2005	Pfizer/Valeant Pharmaceuticals®	NCT00605280, NCT00549055
ACT-GRO-777 (AS1411)	Acute myeloid leukemia, kidney cancer, advanced solid tumors	NCL	Phase II, terminated	Advanced Cancer Therapeutics®	NCT01034410, NCT00740441

ARC1779	Acute myocardial infarction, thrombotic microangiopathy	von Willebrand factor	Phase II, terminated	Archemix®	NCT00507338, NCT00632242, NCT00726544
Ribozyme					
Angiozyme	Kidney cancer	*FLT1*	Phase II	Ribozyme Pharmaceuticals (now Merck®)	NCT00021021
Heptazyme	HCV infection	HCV IRES	Terminated	Ribozyme Pharmaceuticals (now Merck®)	N.A.
Decoy					
STAT3 decoy	Head and neck cancer	*STAT3*	Phase 0, completed	University of Pittsburgh®	NCT00696176
RNA cocktail					
pHIV7-shI-TAR-CCR5RZ	Acquired immunodeficiency symptom	Decoy targeting *TAR*, shRNA targeting *Tat/rev*, and ribozyme targeting *CCR5*	Phase 0	City of Hope® National Medical Center	NCT01153646

CMV, cytomegalovirus; HCV, hepatitis C virus; VEGF, vascular endothelial growth factor; NCL, nucleolin; TTR, transthyretin; TAR, transactivation response element; PKN3, protein kinase N3; GM-CSF/CSF2, granulocyte macrophage colony-stimulating factor; STAT3, signal transducer and activator of transcription 3.

amyloidotic polyneuropathy (NCT01960348). Patisiran silences *TTR* mRNA transcripts to reduce accumulation of amyloid deposits in the liver. The proof-of-concept phase I clinical study of patisiran, performed in unaffected human volunteers, achieved rapid, robust, and durable knockdown of serum TTR after a single dose (Coelho et al., 2013). In the phase II multiple-dose study, the therapeutic was found to be very well tolerated and showed a similar level of serum TTR knockdown as was observed in the phase I study (Coelho et al., 2013). A phase III trial is ongoing and is designed to evaluate the efficacy and safety of the therapeutic in ATTR patients in a randomized, double-blind, and global setting (NCT01960348). If successful, this phase III study will be a significant milestone for the entire field of RNAi therapeutics and will provide a model for future trial design and strategies for RNAi commercialization.

In addition to patisiran, ALN-TTRsc, also from Alnylam® Pharmaceuticals, is another promising lead that targets the same gene, but uses an in-house, proprietary, sugar-based N-acetylgalactosamine (GalNAc) technology, instead of lipid nanoparticles, for systemic delivery (Table 1). The GalNAc delivery technology is generally thought to have a wider therapeutic index than lipid-based nanoparticles and is more targeted to the liver (Manoharan). Moreover, another renowned RNAi therapeutic company, Dicerna Pharmaceuticals®, has developed 27-mer Dicer substrate siRNAs (dsiRNAs) that mimic the substrate for the Dicer processing event. In targeting the RNAi pathway at the initiation point, dsiRNA strategies improve RNAi silencing efficiency compared to the canonical the 21-mer siRNA molecule (Kim et al., 2005). A phase I study has been recently launched to test the dsiRNA therapeutic DCR-MYC in hepatocellular carcinoma (NCT02110563) (Table 1). Furthermore, Silence Therapeutics® has promoted a series of clinical trials of siRNA therapeutics using lipid carriers. Atu027 targets the protein kinase N3 *PKN3* mRNA for the treatment of advanced pancreatic and head and neck cancers (Aleku et al., 2008; Strumberg et al., 2012) (Table 1). The siRNA is heavily modified with a 2′-*O*-methyl sugar to extend its half-life and complexed with cationic liposomes for efficient cell internalization. Atu027 has been tested in phase Ib/IIa trials for safety and efficacy and has been evaluated as an adjunct therapy with GEMZAR® (gemcitabine), a chemotherapeutic drug, for metastatic pancreatic cancers (NCT01808638).

Although the majority of siRNA therapeutics is delivered to cells by biocompatible materials such as GalNAc, delivery of siRNA therapeutics to ex vivo cells via viral or bacterial vectors is also common. Ex vivo is a

term to describe a procedure that takes place outside of an organism. In this case, cells are collected from the patient, modified with gene therapy, and then infused back into the patient. Gradalis® Inc. is developing the FANG™ vaccine, which expresses an mRNA that encodes recombinant granulocyte macrophage colony-stimulating factor (GM-CSF/CSF2) and a bifunctional shRNA (bi-shRNA) targeting *FURIN* (Table 1). The GM-CSF/CSF2 protein works by promoting antigen presentation and the bi-shRNA reduces FURIN production, thereby inhibiting the formation of the mature immunosuppressive TGFβ1 and TGFβ2 isoforms. The bi-shRNA consists of two stem-loop structures with a miR-30a backbone. The guide strand of the bi-shRNA is complementary to the *FURIN* mRNA transcript and induces mRNA degradation, while the passenger strand of the bi-shRNA is mismatched with the target 3′ untranslated region (UTR) and functions as a miRNA (Rao et al., 2010). A phase I study of the FANG™ vaccine demonstrated that TGFβ1 and β2 were downregulated and that the vaccine was safe and elicited an immune response correlated with prolonged survival (Senzer et al., 2012). Several phase II clinical trials are ongoing to test this vaccine in patients with ovarian cancer, advanced melanoma, and colorectal cancer (NCT01551745, NCT01309230, NCT01061840).

2.2 Antisense Oligonucleotides

Historically, the effects of RNA antisense technology have often been confused with those of RNAi-based therapeutics because they both function as gene suppressors (Scherer & Rossi, 2003). Some groups have argued that RNAi is one of the antisense mechanisms because the guide strand of the siRNA/shRNA binds to the target by complementary base pairing. Regardless of the controversies, the generally agreed upon difference is that RNAi-based therapeutics are double-stranded RNAs that recruit the RISC complex to mediate gene silencing, whereas antisense therapeutics are single-stranded oligonucleotides that block target protein translation by either physically blocking the mRNA from binding to ribosomes or by promoting RNase H-mediated mRNA degradation (Lavery & King, 2003). In terms of their design, antisense therapeutics are short (12–21 nucleotides) DNA- or RNA-like compounds that can hybridize to complementary, protein-coding mRNA transcripts to block protein translation (Helene, 1991). When an antisense therapeutic binds to its mRNA target, RNase H recognizes the DNA/RNA hybrid and cleaves the mRNA, which in turn halts production of the protein encoded by that mRNA (Walder & Walder, 1988). Antisense therapeutics could also inhibit gene expression by

altering mRNA splicing or arresting translation initiation (Boiziau et al., 1991; Munroe, 1988).

Single-stranded, naked antisense oligonucleotides are extremely unstable *in vivo* because of the plethora of nucleases in serum and cells. Therefore, chemical modifications that increase oligonucleotide stability can improve their therapeutic properties, such as pharmacokinetics (PK) and pharmacodynamics (PD), without sacrificing potency. Common modifications to synthetic oligonucleotides include a change in sugar (e.g., 2′-*O*-methyl-, 2′-fluoro- or 2′-*O*-methoxyethyl-oligonucleotides) or phosphate backbone (e.g., phosphorothioate, locked nucleic acid (LNA), or morpholino oligonucleotides) (Kurreck, 2003). In the clinical setting, Sarepta Therapeutics® (formerly known as AVI BioPharma, Inc.) developed two antisense phosphorodiamidate morpholino oligomers (PMOs) that are currently being tested in phase I or II clinical trials: eteplirsen (AVI-4658) for Duchenne muscular dystrophy (DMD) (NCT01396239) and AVI-7100 for influenza (NCT01747148) (Table 1). It is thought that the PMO technology, because it is neutrally charged, reduces off-target interactions with cellular proteins and prolongs activity in the body (Popplewell, Malerba, & Dickson, 2012). Same as eteplirsen (AVI-4658), drisapersen from Prosensa Therapeutics® is intended for 13% of all DMD patients by inducing exon 51 skipping in the dystrophin gene (Flanigan et al., 2014). However, the phase III trial result of drisapersen fails to meet the primary end point because there are no statistically significant or clinically meaningful treatment differences between the treatment group and placebo at a dose of 6 mg/kg/week after 48 weeks. Additionally, Isis Pharmaceuticals®, a powerhouse in the antisense therapeutic arena, has several promising products on the market or in late-stage clinical trials. KYNAMRO® (mipomersen sodium) is a United States Food and Drug Administration (FDA)-approved antisense therapeutic that targets a cholesterol-lowering gene, apolipoprotein B, and is used to combat homozygous familial hypercholesterolemia (Hair, Cameron, & McKeage, 2013; Mearns, 2012; Raal et al., 2010) (Table 1). KYNAMRO® contains multiple chemical modifications, including a phosphorothioate backbone and 2′-*O*-methoxyethyl-modified sugars to increase stability and half-life in serum (Ito, 2007). Vitravene (fomivirsen) is another FDA-approved antisense therapeutic developed by Isis Pharmaceuticals®; it became the first approved antisense therapeutic in 1998 (Bonn, 1996). Vitravene contains 21 nucleotides with phosphorothioate linkages and it blocks human cytomegalovirus (CMV) protein synthesis. It is indicated for intravitreal treatment of CMV retinitis in patients

with AIDS (Flores-Aguilar et al., 1997). However, Vitravene was discontinued in the United States because it was unprofitable (Krieg, 2011). Intriguingly, Isis Pharmaceuticals® is also developing an antisense therapeutic (ISIS-TTR_{RX}) to treat TTR amyloidosis, which is the same disease pursued by Alnylam® Pharmaceuticals using siRNA technology (Table 1). Similar to the Alnylam® Pharmaceutical strategy, ISIS-TTR_{RX} inhibits the production of all forms of TTR amyloidosis and has achieved a 75–90% reduction in protein levels in a phase I study (Ackermann et al., 2012). A phase III clinical study is ongoing to evaluate the efficacy of the antisense therapeutic in patients with familial amyloidotic polyneuropathy. On the other hand, in a study conducted by Alnylam® Pharmaceuticals that compared ALN-TTRsc with an antisense oligonucleotide against TTR, Alnylam® Pharmaceuticals claimed that ALN-TTRsc achieved a more rapid, potent, and robust knockdown of the TTR gene than the antisense oligonucleotide targeting TTR, with more than 100-fold lower exposure in "bystander" tissues, such as the kidney (Butler, 2014). However, it is unclear whether there is any difference between the anti-TTR antisense therapeutic used in the Alnylam® Pharmaceuticals study and ISIS-TTR_{RX}.

Although most antisense therapeutics have been designed to target mRNAs, antisense technology can also be deployed to inhibit endogenous miRNAs. miRNAs are another class of naturally occurring ncRNAs (20–25 nucleotides in length) that regulate target gene expression through sequence-specific hybridization to the 3′ UTR of mRNA transcripts. miRNAs play an integral role in many biological processes and dysregulation of this class of ncRNAs has been linked to many diseases, including cancer (Esteller, 2011). Regulus Therapeutics™, the miRNA-based joint venture between Alnylam® Pharmaceuticals and Isis Pharmaceuticals®, leverages both antisense and RNAi technology to develop antisense inhibitors of miRNAs called antagomirs (Krutzfeldt et al., 2005). RG-101 is an antagomir targeting miRNA-122, which is the most abundant miRNA in the liver hepatocytes and is required for maintenance of hepatitis C virus (HCV) infection (Table 1). The antagomir therapeutic is conjugated with GalNAc for systemic delivery and is intended to treat HCV. Additionally, Santaris Pharma A/S® focuses on antisense technology, but uses an in-house LNA drug platform to synthesize antisense therapeutics for targeting mRNAs or antagomirs for targeting miRNAs. Compared with typical nucleic acids, the LNA strategy could improve the affinity between the antisense therapeutic and the RNA target, thereby facilitating cellular delivery and lowering the dose required to achieve the optimal therapeutic index.

miravirsen is the first miRNA-targeted therapeutic to enter clinical trials and is an LNA-modified antagomir targeting miR-122 for combating HCV infection (the same indication as RG-101) (Gebert et al., 2014) (Table 1). A completed phase IIa trial in treatment-naïve patients with chronic HCV genotype 1 infection indicated that the therapeutic is safe, well tolerated, and suppresses HCV resistance (Janssen et al., 2013).

2.3 Aptamers—Chemical Antibodies

One of the main functional pillars of RNA is molecular recognition. Although DNA and RNA exist as a pair of chemical cousins, RNA contains a reactive 2′ OH group on its ribose sugar that can adopt complex structures and allows its bases to form hydrogen bonds with other molecules. Based on these distinguished molecular recognition characteristics, structured RNAs with defined functions could be artificially evolved in a test tube and made to bind any molecular targets with high specificity and affinity. An RNA molecule created in this manner is called an "aptamer," a name derived from the Latin word *aptus* meaning fitting (Ellington & Szostak, 1990). The elegant experimental procedure used to identify a desired aptamer is called systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk & Gold, 1990). Using SELEX, it is possible to select, amplify, and evolve high binding affinity aptamers from a copious amount of a randomized combinatorial nucleic acid library. A typical SELEX library comprises $\sim 10^{14}$–10^{18} sequences. These sequences contain a random region composed of 25–70 nucleotides in their centers. Thus, SELEX libraries provide a pool of molecular and structurally diversified candidates for screening competent and bioactive aptamers that specifically bind the target (Ellington & Szostak, 1990). The random region is flanked by a fixed sequence ($\sim$20 nucleotides) at both ends that is used for polymerase chain reaction (PCR) amplification during each round of SELEX. The SELEX cycle consists of iterative application of four critical steps: (1) incubation of the library pool and the target, (2) partition of target-bound aptamers, (3) recovery of target-bound aptamers, and (4) amplification of recovered aptamers by PCR (Shum, Zhou, & Rossi, 2013). The random pool of nucleic acids screened in this manner usually yields sequences that bind with extraordinary strength and exquisite specificity. A successful SELEX experiment typically requires 12–18 cycles and takes 3–4 weeks, depending on the partition methods, chemistry of the nucleic acids, and size of the library.

Famously called "chemical antibodies," aptamers have many attributes that make them attractive therapeutic agents that rival small molecules and

protein biologics. Relative to other therapeutics, aptamers have higher affinity and specificity, easier manufacturing scale-up, easier chemical modification, better tissue and cell penetration, and lower regulatory barriers (Bunka & Stockley, 2006). Since its inception in 1990, aptamer technology has been tirelessly exploited as an invaluable tool for diagnosis, detection, drug delivery, and particularly therapeutics. In 2005, MACUGEN® (pegaptanib sodium), a treatment for wet age-related macular degeneration, was the first FDA-approved aptamer therapeutic, an important milestone in the field (Table 1) MACUGEN® is marketed by Valeant Pharmaceuticals® in the US and Pfizer® Pharmaceuticals outside the US. The molecular target of MACUGEN® is vascular endothelial growth factor isoform 165 ($VEGF_{165}$), which is an extracellular protein known to mediate ocular neovascularization. Using SELEX, the unique sequence of MACUGEN® was identified and modified chemically to improve its PK and PD. Subsequently, it was shown that MACUGEN® can selectively bind to $VEGF_{165}$ via its heparin binding domain but does not bind other VEGF isoforms. Thus, MACUGEN® can inhibit pathological neovascularization while leaving normal physiological neovascularization unaffected (Ng et al., 2006). In a binding and specificity assay, MACUGEN® bound to $VEGF_{165}$ with a dissociation constant (K_D) value of 140 pM, PDGFA/B with a K_D of 91 nM, FGF2 with a K_D of 286 nM, and thrombin (F2) with a K_D of 3060 nM (Green et al., 1995; Klussmann, 2006). MACUGEN® was administered by intravitreal injections every 6 weeks and exerted its effect by interfering with the binding of VEGF to its receptors fms-related tyrosine kinase 1/VEGF receptor 1 (FLT1) and kinase insert domain receptor/VEGF receptor 2 (KDR), located on the vascular endothelial cell surface (Zuker, 2003). Clinical data showed that MACUGEN® was safe at all doses and that it significantly attenuated vision loss by approximately 50% (Cunningham et al., 2005).

Since the original MACUGEN® studies, many aptamers have been clinically validated. One notable lead is ACT-GRO-777 (previously known as AS1411 prior to acquisition by Advanced Cancer Therapeutics®, Inc.) (Table 1). ACT-GRO-777 is a 26 bp G-quadruplex DNA aptamer that binds to nucleolin, which is a protein commonly found on the surface of cancer cells (Bates, Laber, Miller, Thomas, & Trent, 2009). ACT-GRO-777 was not created using SELEX, but was similarly derived—it was a product of using the "purine motif" design to alter gene expression (Bates et al., 2009). The phase I and II human clinical trials are complete, and they show anticancer activity and a favorable safety profile (Mongelard & Bouvet,

2010; Rosenberg et al., 2014). Recent research has extended the potential use of ACT-GRO-777 to include use as a vector for the delivery of chemotherapeutics and other RNA-based therapeutics (Kotula et al., 2012; Lale, Aswathy, Aravind, Kumar, & Koul, 2014). Moreover, Archemix Corporation launched a few experimentally successful aptameric therapeutics (e.g., ARC1779 targeting von Willebrand factor and ARC19499 targeting tissue factor pathway inhibitor) (Jilma-Stohlawetz, Gilbert, Gorczyca, Knobl, & Jilma, 2011; Waters et al., 2011). However, the company is no longer in business. Aptamer candidates in their R&D pipeline have been either discontinued or licensed to other companies.

2.4 Ribozymes—Catalytic RNAs

Akin to proteins, ribozymes can catalyze chemical reactions and function like molecular scissors, but ribozymes are made of ncRNA molecules. Many known ribozymes catalyze the hydrolysis of phosphodiester DNA bonds in *cis* (the same nucleic acid strand) or *trans* (the opposite strand). In addition to the catalytic domain, ribozymes contain a substrate recognition domain that determines site-specific cleavage. There are many types of ribozymes in nature and they can also be artificially engineered. Naturally existing ribozymes include RNaseP, peptidyl transferase 23S RNA, hammerhead ribozymes, hairpin ribozymes, and many more (Kumar & Ellington, 1995). Most of these ribozymes catalyze RNA cleavage and ligation. Conversely, artificial ribozymes are evolved by *in vitro* selection method that resembles the aptamer SELEX process and is tailored with desirable properties for therapeutic and diagnostic applications (McGinness, Wright, & Joyce, 2002). Some of these ribozymes contain novel structures, but most contain the naturally occurring hammerhead and hairpin motifs. Similar to other RNA therapeutics, ribozymes are highly susceptible to degradation and require certain chemical modifications to improve their stability and pharmacological profiles. Common modifications include inclusion of a phosphorothioate RNA backbone, and the addition of 2′-*O*-methyl and 2′-deoxy-2′-*C*-allyl uridine (Burnett & Rossi, 2012). In terms of delivery, ribozymes can be either delivered to the target cells without any vector or can be constitutively expressed from cDNA loaded in the viral vector. Most often, the effectiveness of the ribozyme technology depends on target accessibility.

Ribozyme Pharmaceuticals was the major biotechnology company developing therapeutic ribozymes in late 1990s. However, with the emergence of RNAi therapeutics in the new millennium, the investors and

company leadership shifted their focus from ribozymes to siRNAs, and Ribozyme Pharmaceuticals was renamed Sirna Therapeutics in 2003, and was later acquired by Merck® Pharmaceuticals in 2006 for $1.1 billion (Krieg, 2011). There is currently diminishing optimism for the promise of ribozyme therapies; however, there are a few important studies on the therapeutic use of ribozymes. One notable example is angiozyme (RPI.4610), which cleaves *FLT1* mRNA to block kidney cancer angiogenesis and tumor growth. A phase I study demonstrated excellent safety and tolerance of angiozyme at doses of up to 300 mg/m^2 in multiple subcutaneous injections (Weng et al., 2005). A phase II efficacy trial was conducted in patients with metastatic breast cancer (Table 1). Although angiozyme was well tolerated, it did not promote any clinical benefit to patients (Morrow et al., 2012). Similarly, Heptazyme (LY466700), also developed by Ribozyme Pharmaceuticals, is another synthetic ribozyme therapeutic for HCV infection. Although it completed phase I/II trials (Usman & Blatt, 2000), the development of Heptazyme was halted because of the failure to meet the clinical end point (Usman & Blatt, 2000) (Table 1).

2.5 RNA Decoys

Another group of ncRNAs used in gene therapy is the RNA decoys. These RNA molecules function like "sponges" to sequester their targets. Most often, the artificial sequence of the RNA decoy is designed to match the natural RNA sequence, which is a target of an RNA-binding protein, such as a transcription factor. Once the decoy is in the cytoplasm, the RNA-binding protein would bind to the RNA decoy. If a copious amount of decoy is present in the cell, then the RNA-binding protein would bind to the synthetic RNA decoy, leaving the natural RNA sequence unbound.

One successful example of an RNA decoy is the transactivation response element (*TAR*) decoy for HIV treatment. In HIV-1, the transactivator of transcription (Tat) protein is a critical regulator of HIV-1 replication and mediates the transcriptional activation of the HIV-1 long terminal repeat by binding to *TAR* in a complex with cellular cyclin T1 in the nucleolus (Garcia et al., 1989). The *TAR* decoys that mimic the apical loop of the HIV *TAR* RNA render the HIV-1 Tat protein incapable of binding to the actual *TAR*, thereby blocking viral transcription and inhibiting HIV-1 replication (Lisziewicz et al., 1993; Michienzi, Li, Zaia, & Rossi, 2002). The *TAR* decoy is currently being tested along with a hammerhead ribozyme targeting *CCR5* and a shRNA targeting *Tat/rev* in a pilot feasibility phase 0 study for patients with AIDS-related non-Hodgkin's lymphoma.

This work is a collaboration between City of Hope® National Medical Center and Benitec Biopharma® (Anderson et al., 2007; DiGiusto et al., 2010; Li et al., 2005) (Table 1). The cocktail of RNA therapeutics (pHIV-7-shI-TAR-CCR5RZ) expressed by separate RNA polymerase III promoters was packaged in a replication-incompetent lentiviral vectors and then transduced ex vivo into $CD34^+$ hematopoietic progenitor cells (DiGiusto et al., 2010). The therapy was well tolerated, and the expressed RNA products were detected in primary blood mononuclear cells of all cohorts up to 6 months after treatment (Burnett et al., 2011).

Furthermore, a double-stranded decoy targeting signal transducer and activator of transcription 3 (*STAT3*) is being tested in a phase 0 study at The University of Pittsburgh for patients with head and neck tumors (Sen et al., 2012) (Table 1). The 15 nucleotide *STAT3* decoy sequence is derived from the *STAT3* response element in the *c-FOS* promoter (Sen et al., 2014). The decoy was modified by cyclization using hexaethylene glycol linkers on both 5′ and 3′ ends. When the circular *STAT3* decoy was injected into the tumor and entered cells via endocytosis, the decoy competitively bound to the phosphorylated, dimeric, cellular *STAT3*, resulting in decreased cell proliferation, survival, and differentiation (Leong et al., 2003).

The decoy approach, proposed by Nobel Laureate Philip Sharp, takes advantage of miRNA regulation. A series of tandemly arranged miRNA target sequences are packaged and expressed by a viral vector, allowing them to function as a sponge to sequester miRNAs and deregulate the miRNA targets (Ebert & Sharp, 2010). It is noteworthy that the optimal number of miRNA target site in the miRNA sponge is 6–12, although it is generally believed that the miRNA suppression efficiency increases with the number of miRNA target sites (Bak, Hollensen, & Mikkelsen, 2013; Ebert, Neilson, & Sharp, 2007). Another example of miRNA decoys is the "Tough Decoy" (TuD) hairpin. TuD is a ~60 nucleotide long, hairpin-shaped RNA with an internal loop exposing two miRNA target sites (Hollensen, Bak, Haslund, & Mikkelsen, 2013). The miRNA-binding sites are not perfectly complementary to the target miRNA sequences, but create a central bulge so as to bypass argonaute RISC catalytic component 2 (AGO2)-mediated cleavage and confer resistance to serum nucleases (Hollensen et al., 2013). As a result, the miRNA expression level is reduced by the dual-targeting TuD. Since miR-122a is a well-studied miRNA exclusively expressed in the liver, potent miRNA-122 suppression in HCV has been observed when an adeno-associated vector encodes a

TuD targeting miR-122a (Sakurai et al., 2012). Intriguingly, when researchers compared the miRNA suppression capacity of the TuD and the sponge decoy containing eight miRNA target sites, the TuD carrying two miRNA target sites was as potent and, for some miRNAs more potent, than the sponge decoy carrying several target sites (Bak et al., 2013). Collectively, these miRNA decoys definitely require specialized delivery formulations (e.g., viral vector) and are being tested in animal models. It remains to be seen how these structured miRNA inhibitors will work in human trials.

2.6 Bacteriophage phi 29 RNA

In nature, long ncRNA molecules use helical base pairing along with loop–loop base pairing to form extremely stable RNA nanoscale structures. These nanoparticles display an amazing diversity in function and versatility in structure. One glowing example is the bacteriophage phi 29 RNA (pRNA)-packaging motor, which is necessary for the bacteriophages to load their 9 kb viral genomic DNA into the viral capsid (Guo, 2010). Structurally, the pRNA-packaging motor is made up of six pRNA fragments resembling a group of six people standing in a circle and each fragment is >120 nucleotides long and contains two independent domains: (1) a helical domain and (2) an interlocking domain (Chen, Zhang, & Guo, 1999; Guo, Zhang, Chen, Garver, & Trottier, 1998). The helical domain contains a very strong and stable helical structure, while the interlocking domain of each pRNA contains two loops that help connect to other pRNAs to form a hexameric packaging motor through the formation of canonical complementary bp (Shu, Huang, Hoeprich, & Guo, 2003). Therefore, the pRNA fragment acts like a Lego® brick and can be teamed up to build various pRNA nanostructures in which the helical domain of each fragment could be decorated with a functional unit, such as a siRNA, aptamer, antisense oligonucleotide, or ribozyme. Then, the interlocking domain of the pRNA could be artfully crafted to assemble pRNAs into dimers, trimers, and hexamers by simple incubation under ambient conditions. This structure allows for powerful flexibility to design complicated future therapeutics.

Given the flexibility and versatility in the design format, Peixuan Guo's research group and others have engineered a set of pRNA nanoparticles for gene therapy. In one example, folates, which are cancer cell-targeting ligands, were attached to the helical domain of one pRNA fragment and then connected to another pRNA fragment conjugated to an anti-survivin siRNA (Tarapore, Shu, Guo, & Ho, 2011). Thus, the attached folate

molecule can escort the dimeric nanoparticle into cancer cells that overexpress folate receptors on the surface. In a preclinical study, dimeric pRNA/folate/siRNA nanoparticles ranging from 10 to 50 nm were optimal for efficient cancer cell internalization and promoted apoptosis (Tarapore et al., 2011). Moreover, our group created novel pRNA nanoparticles by adding cell-targeting aptamers for both cell-specific targeting and efficient siRNA delivery (Zhou, Shu, Guo, Smith, & Rossi, 2011). A dimeric gp120 aptamer-pRNA-*Tat/rev* siRNA nanoparticle was constructed and tested in the HIV-1 infection model. It was demonstrated that both the gp120 aptamer-pRNA and the *Tat/rev* siRNA-pRNA dimerized and subsequently internalized into HIV-1-infected cells expressing gp160 and inhibited HIV-1 replication (Zhou et al., 2011). These early studies indicated that the attachment of an RNA functional motility does not interfere with the formation of multimeric nanoparticles.

Although there are nearly limitless ways to imagine therapeutics being loaded onto the pRNA nanoparticle, manufacturing of the nanoparticle is difficult because the capacity to synthesize lengthy modified RNA oligonucleotides with high yield and purity is not readily available in either academic or commercial settings. With this challenge in mind, Guo and colleagues first investigated the root cause of the ultrastable nature of pRNA. They found that the centerfold domain between the helix domain and the interlocking domain confers pRNA stability. Later, the centerfold domain was engineered to form a T-shaped Three-way junction pRNA (Shu, Shu, Haque, Abdelmawla, & Guo, 2011) and an X-shaped four-way junction pRNA (Haque et al., 2012; Shu et al., 2013). In both cases, the nanoparticles were incredibly stable, even in denaturing conditions, easy to manufacture on a large scale and multivalent, meaning that multiple functional modules (e.g., aptamers, siRNAs, ribozymes, chemical ligands, and antisense oligonucleotides) could be joined together within the nanoplatform and operated independently. In the four-way junction pRNA example, the four ends of the X-shaped nanoparticles were decorated with a malachite green aptamer (for detection), a folate molecule (for targeted delivery), a *survivin* siRNA (for gene silencing), and a *luciferase* siRNA (for functional testing) (Haque et al., 2012). This tetravalent nanoparticle was thermodynamically intact, stable at low concentrations and resistant to urea denaturation (Haque et al., 2012). Systemic tail vein injection of the nanoparticle into mice demonstrated that the nanoparticle stayed bound to the cancerous tissue for more than 8 h without affecting other healthy tissues or organs (Haque et al., 2012). Although the use of pRNA for gene therapy looks promising in

animal models, there is no pRNA-based therapeutic in the human clinical pipeline yet.

3. CHALLENGES FOR SUCCESSFUL GENE THERAPY USING NONCODING OLIGONUCLEOTIDES

Interest in the future of noncoding oligonucleotides for gene therapy is very high. Across all types discussed in this chapter and in the literature, proof-of-concept *in vitro* and animal tests have confirmed the capabilities of these oligonucleotides to effectively treat diseases including the following: siRNAs/shRNAs to silence gene expression, antisense oligonucleotides to block protein translation, aptamers to inhibit protein function, ribozymes to cleave mRNA transcripts, RNA decoys to compete with natural targets, and pRNAs to build nanoparticles. An influx of investors into the oligonucleotide therapeutic sector has allowed several nucleic acid therapeutic developers to launch successful initial public offerings in early 2014 and has enabled the companies to begin human clinical testing of lead candidates (Bouchie, 2014). Despite early enthusiasm, sound scientific basis, and enormous monetary investment, there are only a few nucleic acid-based therapeutics in the clinic, and none of them is likely to become a blockbuster (annual sales > $1 billion) that can challenge the market share of the traditional therapies. There are several reasons why nucleic acid therapeutics are not yet ready for a routine form of therapy. The most important and most difficult problems are the efficiency and specificity of delivery. The therapeutic must evade the reticuloendothelial system as it circulates after administration, and also overcome the barrier of the endosome, and avoid degradation before arriving at its site of action in the target cells (Zhang, Satterlee, & Huang, 2012). This issue has raised concerns within the field from the beginning and resulted in several discontinuations of clinical trials due to insufficient efficacy of the therapeutic strategy in humans. For example, Allergan® discontinued development of the AGN-745 siRNA, which targets VEGF in patients with wet age-related macular degeneration, because the treatment did not improve the patients' vision in a phase II study (Burnett & Rossi, 2012; Burnett et al., 2011). Similarly, Tekmira® delayed their TKM-ApoB clinical development program in 2010 despite a major investment in animal testing (Burnett et al., 2011). As such, the future success of oligonucleotide therapeutics relies on overcoming the challenges of specific, robust delivery and reviving enthusiasm for this promising class of therapeutics.

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CONFLICT OF INTEREST STATEMENT

JJR is a scientific cofounder at Dicerna Pharmaceuticals®, an investigator at Benitec Biopharma®, and an investigator of the clinical trial program (NCT01153646) conducted at City of Hope National Medical Center, Duarte, CA.

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CHAPTER SEVEN

Self-Amplifying mRNA Vaccines

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Contents

Abstract

This chapter provides a brief introduction to nucleic acid-based vaccines and recent research in developing self-amplifying mRNA vaccines. These vaccines promise the flexibility of plasmid DNA vaccines with enhanced immunogenicity and safety. The key to realizing the full potential of these vaccines is efficient delivery of nucleic acid to the cytoplasm of a cell, where it can amplify and express the encoded antigenic protein. The hydrophilicity and strong net negative charge of RNA impedes

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cellular uptake. To overcome this limitation, electrostatic complexation with cationic lipids or polymers and physical delivery using electroporation or ballistic particles to improve cellular uptake has been evaluated. This chapter highlights the rapid progress made in using nonviral delivery systems for RNA-based vaccines. Initial preclinical testing of self-amplifying mRNA vaccines has shown nonviral delivery to be capable of producing potent and robust innate and adaptive immune responses in small animals and nonhuman primates. Historically, the prospect of developing mRNA vaccines was uncertain due to concerns of mRNA instability and the feasibility of large-scale manufacturing. Today, these issues are no longer perceived as barriers in the widespread implementation of the technology. Currently, nonamplifying mRNA vaccines are under investigation in human clinical trials and can be produced at a sufficient quantity and quality to meet regulatory requirements. If the encouraging preclinical data with self-amplifying mRNA vaccines are matched by equivalently positive immunogenicity, potency, and tolerability in human trials, this platform could establish nucleic acid vaccines as a versatile new tool for human immunization.

1. INTRODUCTION

Due to their potential in combining the positive attributes of both live-attenuated and subunit vaccines, there has been substantial scientific effort in developing nucleic acid-based vaccines, over the last 20 years. Plasmid DNA (pDNA) and viral vectors have been extensively evaluated as vaccines in human clinical trials. Although each of these platforms has demonstrated some advantages, no vaccine using these technologies, has been licensed for human use (Kutzler & Weiner, 2008; Liu, 2011; Sardesai & Weiner, 2011). More recently, mRNA-based vaccines have emerged as potential alternatives and may offer certain advantages over their DNA-based counterparts (Deering, Kommareddy, Ulmer, Brito, & Geall, 2014; Geall, Mandl, & Ulmer, 2013; Kallen et al., 2013). Historically, this approach has been overlooked due to skepticism regarding mRNA stability and the perception that the barriers in large-scale commercial production were insurmountable. The objectives of this chapter are to: (1) provide a brief introduction into the various types of nucleic acid-based vaccines, (2) introduce the most recent research efforts in using nonviral systems to deliver self-amplifying mRNA vaccines, (3) summarize how the barriers of large-scale production and mRNA stability have been overcome, (4) highlight the preclinical data and potential disease targets that self-amplifying mRNA vaccines might be used to address, and (5) review the future prospects for this emerging vaccine technology.

2. HISTORY OF NUCLEIC ACID VACCINES

Since the beginning of the vaccine era, with the introduction of the smallpox vaccine more than two centuries ago, live-attenuated vaccines, programed by their nucleic acid genomes, have been effectively used in preventing infectious diseases. The most recent versions of smallpox vaccines are based on a live, replicating bovine pathogen (vaccinia virus) that confers protective immunity against disease caused by a related human pathogen (variola virus), and is safe for use in humans. The basis of protective immunity from live attenuated viral vaccines is now believed to be an induction of crossreactive antigen-specific antibody and T cell responses (Wrammert, Miller, Akondy, & Ahmed, 2009) as a result of limited viral infection launched by delivery of an intact viral genome. Today, there are several licensed live attenuated vaccines that function in a similar manner, including those that protect against viral (polio, measles, mumps, rubella, influenza, yellow fever, rotavirus, chicken pox) and bacterial diseases (tuberculosis, typhoid fever). However, it is not always possible or practical to use this approach. For example, certain live organisms (e.g., hepatitis C virus (HCV)) do not grow well *in vitro*, making production of such vaccines a challenge. In addition, the potential for reversion of live attenuated organisms to a virulent state poses substantial risks for development of live vaccines against highly lethal pathogens (e.g., Ebola virus). For these reasons, vector-based systems are being developed as a potentially safer alternative to live attenuated vaccines.

Bacterial and viral vectors can efficiently deliver nucleic acids into cells, in a manner similar to delivery by live attenuated organisms. Vector genomes are engineered to express the antigen target(s) of the vaccine. In some cases, the vector may also be engineered to render the construct incapable of replicating and spreading in the immunized host and to remove virulence factors responsible for pathogenicity. Vector-based vaccines are effective due, at least in part, to the efficiency of delivery of the nucleic acid payload using pathways of cellular entry employed by the pathogen. In this way, vectors mimic a live viral or bacterial infection while reducing the safety risks associated with live organisms. In addition, certain vector systems have been used as a platform technology to deliver a wide variety of vaccine antigens. The two most commonly employed platform vectors, vaccinia (Gilbert, 2013) and adenovirus (Johnson, Barouch, & Baden, 2013), have been shown in many human clinical trials to be effectively elicit antigen-specific antibody and T cell responses. However, a significant

limitation of this approach is interference by antivector immunity, either from previous exposure to live infection (e.g., adenovirus) or by prior immunization (e.g., smallpox). Neutralizing antibody responses directed toward the viral proteins present on the surface of the vector or T cell responses against expressed proteins of the vector can blunt the effectiveness of the vaccine and the ability to obtain useful responses from booster doses (Saxena, Van, Baird, Coloe, & Smooker, 2013).

Nonviral delivery of nucleic acids can avoid antivector immunity. pDNA vaccines, which are being developed as an alternative to vector-based vaccines, are simple and lack viral proteins. More than two decades of research and development has amply demonstrated the general utility of pDNA vaccines as a platform to elicit antibody- and T cell-mediated protection in animal models of infectious and noninfectious diseases. Several hundred clinical trials have shown pDNA vaccines to be safe in humans. However, despite intense development efforts, pDNA vaccines have shown insufficient immunogenicity in humans and have required milligram doses of nucleic acid to elicit a response for most intended vaccine applications. One key limitation of pDNA vaccines is inefficient delivery into the nucleus of the target cells, as this is required for transfection and production of the encoded antigen. Various formulations and delivery devices have been evaluated, with electroporation in situ being the most effective (Saade & Petrovsky, 2012). A therapeutic HPV DNA vaccine candidate delivered by electroporation has shown promise in human clinical trials (Bagarazzi et al., 2012), and a cytomegalovirus (CMV) DNA vaccine candidate formulated with a polymer delivery system has an apparent impact against transplant-associated CMV disease (Wloch et al., 2008).

Nonviral delivery of mRNA is also being investigated for nucleic acid-based vaccines, and includes the use of nonamplifying mRNA as well as engineered self-amplifying mRNA derived from viral genomes. As will be discussed in detail in this chapter, mRNA vaccines have the potential to combine many different positive attributes of other vaccines, such as (1) induction of both humoral and cell-mediated immunity (live attenuated vaccines), (2) focused immune responses (subunit vaccines), (3) safety and simplicity (pDNA vaccines), and (4) robust, generic manufacturing methods (pDNA vaccines). In addition, mRNA vaccines can be produced rapidly (within days of obtaining gene sequence information), using completely synthetic manufacturing processes, making them ideal for rapid responses to newly emerging pathogens.

3. INTRODUCTION TO mRNA VACCINES

Presently, there are two major forms of mRNA vaccines, distinguished by the translational capacity of the RNA. They are as follows: (1) small, nonamplifying mRNA molecules that encode the antigen of interest, and (2) larger self-amplifying mRNA molecules that encode an engineered viral replicon from an alphavirus in addition to the antigen of interest (see Figure 1).

Both approaches have certain advantages and limitations. Nonamplifying mRNA molecules used in mRNA vaccines consist of five key elements critical in the life cycle and expression of an mRNA: a cap structure (m^7Gp_3N (N: any nucleotide)), a 5′ untranslated region (5′ UTR) situated immediately upstream of the translation initiation codon; an open reading frame (ORF) that encodes a gene of interest (GOI), a 3′ untranslated region (3′ UTR), and the tail of 100–250 adenosine residues (poly(A) tail) (Figure 1). Sequences in the *cis*-regulatory 5′ and 3′ UTR elements control gene expression by recruiting a variety of cellular proteins to manage translation and degradation rates of mRNA, respectively (Barrett, Fletcher, & Wilton, 2012) (Chatterjee & Pal, 2009; Pickering & Willis, 2005). All five elements in the mRNA construct control the synthesis of proteins by

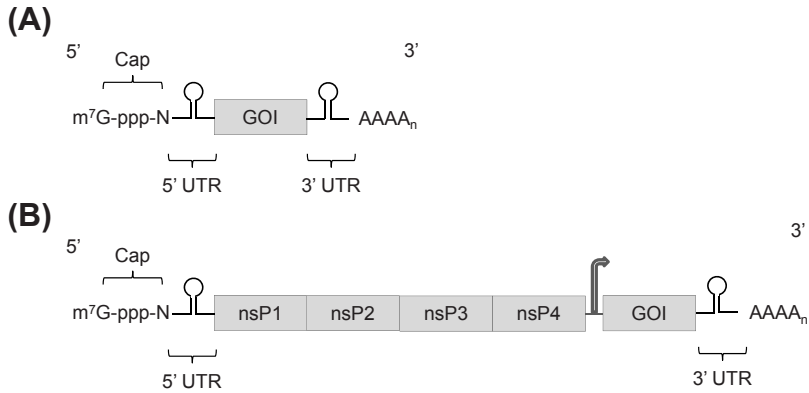

Figure 1 Characterization of nonamplifying mRNA and self-amplifying mRNA vectors. (A) Schematic illustration of a mature eukaryotic mRNA containing a cap structure (m^7Gp_3N (n: any nucleotide)), the 5′ untranslated region (5′UTR), an open reading frame encoding a gene of interest (GOI), the 3′ untranslated region (3′UTR), and a tail of 100–250 adenosine residues (poly(A) tail). (B) Schematic illustration of a self-amplifying mRNA derived from an alphavirus containing a 5′cap, nonstructural genes (nsP1–4), 26S subgenomic promoter (open arrow), the GOI, the 3′ untranslated region (3′UTR), and a poly(A) tail.

influencing mRNA st ability, accessibility to ribosomes, and circularization and interaction with the translation machinery. These elements can be optimized for mRNA vaccine applications and have been reviewed elsewhere (Gingras, Raught, & Sonenberg, 1999; Pascolo, 2008; Ross, 1995). The advantages of using nonamplifying mRNA include the simplicity of the construct, the relatively small size of the RNA compared to a self-amplifying molecule (~two to three versus ~10 kb), and the absence of any additional encoded proteins that could be unintended targets of immune responses (Schlake, Thess, Fotin-Mleczek, & Kallen, 2012). However, due to the short half-life and instability of mRNA, only transient and low level of expression can be achieved *in vivo* by nonamplifying mRNA. Additionally, mRNA is diluted in rapidly dividing cells. In an effort to improve the stability, durability, and expression levels of mRNA-based vaccines, mRNA has been optimized by incorporating specific sequence elements and synthetic, altered ribonucleotides (Kallen et al., 2013).

4. SELF-AMPLIFYING mRNA

Self-amplifying mRNAs are considerably larger (≈9–10 kb) than nonamplifying mRNAs, but also contain the basic elements of mRNA (a cap, 5′ UTR, 3′ UTR, and poly(A) tail of variable length), outlined above (Figure 1). The best studied self-amplifying mRNA molecules are derived from alphavirus genomes, such as those of the Sindbis, Semliki Forest, and Venezuelan equine encephalitis viruses (for review see (Atkins, Fleeton, & Sheahan, 2008; Lundstrom, 2000; Schlesinger, 2001; Smerdou & Liljestrom, 1999). In an alphavirus-based, self-amplifying mRNA, the additional RNA contains a large ORF that encodes four nonstructural proteins (nsP1-4) and a subgenomic promoter. Genes in the viral genome that encode the viral structural proteins are replaced by gene(s) encoding the gene(s) of interest, thereby rendering the mRNA incapable of producing an infectious virus. After delivery into the cytosol of a cell, the released mRNA is translationally competent, and engagement with the host cell ribosome produces the four functional components of RNA-dependent RNA polymerase (RDRP) or viral genome replication apparatus: nsP1, nsP2, nsP3, and nsp4 (Figure 2).

Formation of the RDRP is a complex, multistage process, with each of the nsPs having several functions (for a review see (Shin et al., 2012). Translated nsPs form replication factories on the surface of intracellular membranes and transcribe full-length negative-strand copies from the input

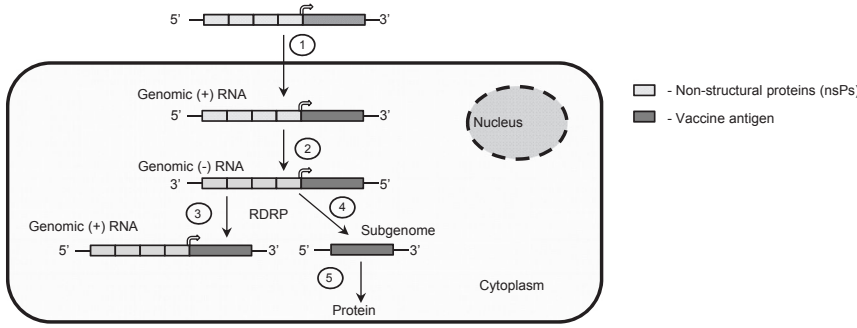

Figure 2 Schematic illustration of the steps in replication and expression of self-amplifying mRNA after delivery to a target mammalian cell. (1) Delivery of mRNA to the cytoplasm. (2) Translation of the open reading frame encoding the four nonstructural proteins (nsP1–4) that form the RNA-dependent RNA polymerase (RDRP), which produces a negative-sense copy of the genome. (3) RDRP catalysis of production of positive-sense genomes from the negative sense copy. (4) RDRP transcription of the subgenome. (5) Translation of the vaccine antigen, leading to protein expression.

mRNA. This negative-strand copy then serves as a template for two positive-strand RNA molecules: the genomic mRNA and a shorter, colinear subgenomic mRNA that corresponds to the 3′ third of the genomic RNA. This subgenomic mRNA (also known as the 26S RNA), is transcribed at extremely high levels, permitting the amplification of mRNA encoding the vaccine antigen. Self-amplifying mRNA vaccines have attractive features that are lacking in nonamplifying mRNA vaccines, such as the autoreplicative ability resulting in high levels of expression of the encoded vaccine antigen in host cells, regardless of cell division. Additionally, the duration of expression from self-amplifying mRNA molecules is enhanced, with expression of nonimmunogenic reporter proteins, such as firefly luciferase, lasting almost two months *in vivo* (Geall et al., 2012). Potential interactions between encoded nsPs and host factors require additional investigation. In laboratory animal studies, immune responses against the nsPs, if elicited, have not been observed as having interfering, antivector effects upon subsequent boosting with a viral replicon particle vaccine (Uematsu et al., 2012).

5. DELIVERY OF SELF-AMPLIFYING mRNA VACCINES

A key challenge in realizing the full potential of self-amplifying mRNA vaccines is the efficient nonviral delivery of the nucleic acid to the cell cytoplasm, where it can amplify and express the encoded vaccine

antigen. Nonviral gene delivery can be achieved in several different ways, including chemical delivery by lipids, polymers, emulsions, or other compounds that facilitate entry into the cells and physical delivery technologies and devices. Most of the reported nonviral gene delivery systems have been developed for pDNA, antisense RNA, siRNA, and mRNA (for a recent review, see (Deering et al., 2014). Delivery of self-amplifying mRNA *in vivo* is a complex multistep process. First, RNA must reach the target tissue while avoiding RNase-mediated degradation and clearance. When it encounters the target cell, RNA must translocate through the cellular membrane to enter the cytoplasm, engage the host translation machinery and initiate translation of the nonstructural proteins. The hydrophilicity and strong net negative charge of RNA significantly impairs cellular uptake. To overcome this barrier, RNA can be electrostatically complexed with cationic lipids or polymers or physically delivered using electroporation or ballistic particle-mediated delivery to improve cellular uptake.

Viruses have evolved sophisticated mechanisms that exploit or circumvent cellular signaling and transport pathways to traffic within host cells and deliver their genome into the appropriate subcellular compartment (Glover, 2012). Therefore, it is worth reviewing viral delivery of self-amplifying mRNA.

5.1 Viral Delivery Systems

Alphaviruses are positive-sense RNA viruses that form the largest genus in the family *Togaviridae*. The alphavirus particle is a 70 nm diameter icosahedron, containing a single-strand, positive-sense RNA genome that is capped and polyadenylated. The genome is approximately 11.5 kilobases in length and is complexed to the capsid protein (C), producing a nucleocapsid core that is enveloped in a cell-derived lipid bilayer embedded with E1 and E2 glycoproteins. Both glycoproteins have transmembrane domains that anchor the spike to the bilayer, and the internal extension of the E2 protein contacts the nucleocapsid core of the virion, thereby orienting the glycoprotein spikes (Kuhn, 2007). The E1 and E2 glycoproteins provide cell targeting and endosomal escape functions to the virus. It was previously thought that alphaviruses enter cells by receptor-mediated endocytosis, followed by a viral membrane, cell membrane fusion event in the endolysosomes to release the RNA and nucleocapsid into the cell cytosol (Kielian & Rey, 2006). However, a growing body of work challenges this model and demonstrates that alphavirus entry is independent of endocytosis and acidic pH-dependent membrane fusion (reviewed in (Brown & Hernandez,

2012; Vancini, Wang, Ferreira, Hernandez, & Brown, 2013), suggesting that viral entry may also occur directly through the plasma membrane.

Several groups have adapted alphaviruses for use as vaccine vectors (Bredenbeek, Frolov, Rice, & Schlesinger, 1993; Liljestrom & Garoff, 1991; Pushko et al., 1997). In the simplest form, this is accomplished by replacing the structural protein genes of an alphavirus with a heterologous GOI. The resulting mRNA, called a replicon, is capable of directing its own replication and heterologous gene expression when introduced into the cytoplasm of host cells; however, since it does not encode the alphavirus capsid or glycoprotein genes, it is incapable of forming virions or spreading to adjacent cells. If these replicons are introduced into a helper cell in which the capsid and glycoprotein genes are expressed in *trans*, virus replicon particles (VRPs) with identical protein and lipid structure to wild-type alphaviruses and that encapsidate replicon RNA in place of a normal alphavirus genome are produced. VRPs are capable of infecting cultured cells and cells in inoculated animals and expressing the encoded GOI; however, they are incapable of producing a viral particle and spreading cell-to-cell due to the lack of structural protein genes in the replicon RNA.

Once an alphavirus enters the cytosol, it coopts the host machinery for translation and expresses an active replicase from its nsP genes. The alphavirus replication machinery and RNA relocalize to immune-privileged "replication complexes" that are typically composed of membrane invaginations into the cytoplasmic face of the cell membrane (Jose, Snyder, & Kuhn, 2009). Within the replication complex, a negative strand complementary RNA intermediate is transcribed from the positive strand genomic RNA, which in turn serves as a template for synthesis of additional genome-length RNAs as well as subgenomic RNAs that encode the structural proteins (in the case of viral infections) or the GOI (in the case of VRPs). Changes in RDRP activity during the replication cycle favors the production of the later RNAs in the late stages of replication, providing a further amplification of the mRNA that encodes the GOI relative to the genome length RNAs that are translated to produce the viral nsPs.

VRP-based vaccine candidates have been extensively tested against bacterial, viral, and protozoan pathogens in a variety of small animal and primate models (reviewed in (Atkins et al., 2008; Rayner, Dryga, & Kamrud, 2002). More importantly, a VRP-based CMV vaccine has been tested in humans (Bernstein et al., 2009). The vaccine consists of two types of replicon particles, one that expresses CMV gB, and a second which expresses a CMV pp65/IE1 fusion protein. The vaccine has been found to be safe and

immunogenic in phase I testing, as neutralizing antibodies against CMV and polyfunctional $CD4^+$ and $CD8^+$ antigen-specific T cell responses to CMV pp65, gB, and IE1 were demonstrated in almost all of the study participants. Although both the preclinical and clinical data for the viral delivery of self-amplifying mRNA are encouraging, this technology requires the use of large-volume electroporation of the genetic elements into cells in culture. At this time, it is unclear whether these methodologies will be amenable in industrial-scale production. In addition, during the production of VRPs in cells containing both replicon and helper RNAs, there is the possibility that recombination or copackaging of replicon and helper RNAs could give rise to infectious viruses.

5.2 Nonviral Delivery Systems

5.2.1 Naked

Initial nonamplifying mRNA immunization experiments were conducted over twenty years ago when intramuscular injection of mRNA in mice was shown to produce its encoded reporter protein *in vivo* (Wolff et al., 1990). The initial proof-of-concept studies indicated that injection of similar doses (on a mass basis) of sucrose-formulated nonamplifying mRNA and pDNA resulted in similar levels of reporter gene expression. Shortly after these initial experiments, Martinon et al. showed that nonamplifying mRNA encoding the influenza virus nucleoprotein encapsulated in liposomes could elicit an antigen-specific immune response (Martinon et al., 1993). In 1994, Peter Liljestrom and his team at the Karolinska Institute in Sweden first demonstrated that naked, self-amplifying mRNA had the potential for use as a nucleic acid vaccine (Zhou et al., 1994). In these pioneering experiments, researchers showed that intramuscular delivery of 10 μg of naked replicon mRNA encoding the influenza nucleoprotein antigen elicited an antibody response, though at a lower titer than elicited by viral delivery of the same RNA. In 1995, Johanning et al. (Johanning et al., 1995) compared inoculation of mice with self-amplifying and nonamplifying mRNA encoding the reporter gene luciferase. A Sindbis virus-derived self-amplifying mRNA, administered by an intramuscular injection into the tongue (intraglossal injection), expressed 24-fold more protein for a fivefold longer duration compared to nonamplifying mRNA.

Since those initial experiments, several groups have investigated the administration of naked self-amplifying mRNA in vaccine applications (for reviews, see (Atkins et al., 2008; Leitner, Ying, & Restifo, 1999; Rayner et al., 2002; Smerdou & Liljestrom, 1999; Zimmer, 2010). Although

injections of naked self-amplifying mRNA elicited immune responses, degradative enzymes in tissues likely limited the amount of unprotected RNA that could be internalized by cells and may explain highly variable and low reporter gene expression and antigen-specific immune responses compared to those following injection of RNA protected by a lipid delivery system (Geall et al., 2012). To overcome the inefficiency and variability of responses to naked RNA, studies have focused on facilitated delivery approaches such as electroporation, gene gun, lipid nanoparticles (LNPs), cationic nanoemulsions (CNEs), and polymers.

5.2.2 Particle-Mediated Epidermal Delivery or "Gene Gun"

Particle-mediated epidermal delivery (PMED) is an efficient, physical method of delivering nucleic acids into cells through the plasma membrane. The device used for this delivery technology is commonly referred to as the "gene gun." Nucleic acids are precipitated onto microparticles, typically 1 μm in diameter and made of gold or tungsten. The nucleic acid-loaded particles are coated on the inner surface of the plastic tubing, which is cut into individual cartridges. These cartridges are loaded into the gene gun, and the gold carrier particles, with their nucleic acid cargo, are transferred by a high pressure helium blast (typically 400 psi, although various pressure settings have been used) directly into the cells. The method can be applied to both cultured cells and *in vivo* in the epidermal layer of the skin. In the skin, nucleic acid is delivered directly into the cytoplasm or nuclei of keratinocytes and Langerhans cells at the site of administration (Fuller, Loudon, & Schmaljohn, 2006). PMED delivery systems have been employed since the mid-1980s for the direct delivery of pDNA for gene therapy and vaccine applications (For a review, see (Dean, Haynes, & Schmaljohn, 2005; Fuller et al., 2006). Immune responses in larger species elicited by intramuscular injection of pDNA have been generally lower than those elicited in small animals, with 1000-fold more pDNA required for effective immunization of larger species than of smaller species (mg versus μg) (Kutzler & Weiner, 2008). However, PMED of DNA vaccines enables significant responses in microgram doses in nonhuman primates (NHPs) (Dean et al., 2005; Fuller et al., 2006, 2002; Payne, Fuller, & Haynes, 2002). In humans, PMED delivery of pDNA was demonstrated to be safe (Payne et al., 2002; Tacket et al., 1999). However, the magnitude of the immune response was modest, administration into multiple sites in the skin was required, and the device was bulky. As a result, the technology was never developed for commercial application or the delivery of prophylactic vaccines.

The gene gun has also been used in a number of preclinical studies in delivering mRNA vaccines, although this approach has been explored much less than in pDNA vaccines, and no clinical studies have been performed to date. Qiu et al. pioneered the field by demonstrating that nonamplifying mRNA encoding human antitrypsin elicited a strong and consistent antibody response in mice, which could be boosted by a second vaccination (Qiu, Ziegelhoffer, Sun, & Yang, 1996). PMED was applied to the delivery of self-amplifying mRNA (Aberle, Aberle, Kofler, & Mandl, 2005; Kofler et al., 2004; Mandl et al., 1998) by Christian Mandl and his team at the University of Vienna in Austria. In these experiments, they showed that PMED delivery of 1 ng of RNA encoding a live attenuated tick-borne encephalitis virus (TBEV) was able to elicit protective immunity after a single dose in mice. PMED delivery of a non-infectious RNA replicon at a dose of 1 μg was sufficient in inducing protective immunity. Immune responses were uniform; there was little variability between individual mice; responses could be increased with a booster immunization; and responses were maintained for at least 1 year and were quantitatively and qualitatively similar to those elicited by a live-attenuated virus. Proof of concept in larger animals, cattle and sheep, was achieved by Vassilev et al., who delivered a full-length infectious clone of bovine diarrhea virus (BVDV) using PMED. In these studies in animals with body weights similar or higher than those of humans, a dose of 0.6 μg of RNA was sufficient in initiating viral replication, leading to an immune response that was indistinguishable from that stimulated by conventional viral infection.

These results demonstrate the utility of PMED-facilitated delivery of self-amplifying RNA vaccines. However, as shown later in the chapter, there are no potency advantages of using this delivery technology rather than lipid-based delivery systems with intramuscular injection by needle and syringe in small animals.

5.2.3 Electroporation (EP)

One of the most promising approaches of overcoming the poor potency of pDNA in larger species is delivery by in situ electroporation (Sardesai & Weiner, 2011). The principal behind EP is simple: by applying a pulsed electrical field that surpasses the electrical capacitance of the cell membrane, cells may be rendered transiently permeable by the formation of hydrophilic pores, driving movement of the negatively charged DNA in the electric field into the cell (Gothelf & Gehl, 2012; Rochard, Scherman, & Bigey, 2011). During the procedure, pDNA is delivered to the cytoplasm of the cell,

and some is subsequently translocated across the nuclear pore. In large animals and humans, intramuscular injection of naked pDNA is accompanied by the insertion of a needle array around the injection site and application of a series of electrical pulses (IM EP). The technology is very effective at increasing gene expression in muscle and enhancing the potency of DNA vaccines in animal models (Sardesai & Weiner, 2011). Recent results from a human clinical trial have shown this approach to elicit functional antibody and Th1-type T cell responses (Bagarazzi et al., 2012). More recently, a less invasive procedure has been described in preclinical models in which the pDNA is administered by intradermal injection followed by application of EP on the surface of the skin (ID EP) (Kichaev et al., 2013; Kulkarni et al., 2013).

Three groups have recently published on the application of EP in delivering self-amplifying mRNA (Cu et al., 2013; Johansson, Ljungberg, Kakoulidou, & Liljestrom, 2012; Piggott, Sheahan, Soden, O'Sullivan, & Atkins, 2009). Piggott et al. were the first to demonstrate that EP could enhance the delivery of self-amplifying mRNA (Piggott et al., 2009). They demonstrated that, with a Semliki Forest virus (SFV) self-amplifying mRNA that IM EP of 50 μg of RNA resulted in β-galactosidase and luciferase reporter gene expression at the site of injection in mice. Some tissue damage was reported after EP delivery, however, when delivered via EP. β-galactosidase was significantly more immunogenic after two doses, as compared to delivery of naked RNA at the same 50 μg dose. Interestingly, viral delivery of the same RNA (10^6 infectious units) produced a comparable response to IM EP, and all immune responses were boosted following the second immunization on day 13. There was a significantly higher IgG2a antibody response with viral delivery and a significantly higher IgG1 response with EP delivery. The skin is an attractive target tissue for vaccination since it contains migrating professional antigen presenting cells and skin-resident dendritic cells. Johansson et al. recently demonstrated that non-invasive ID EP delivery of 5 μg of SFV self-amplifying mRNA elicited strong cellular and humoral immune responses in mice.

In collaboration with Inovio Pharmaceuticals, Novartis Vaccines used the Elgen 1000 EP device to deliver self-amplifying mRNA to muscle. Substantial enhancement of RNA gene expression in situ was observed over naked RNA delivered by IM injection in mice. In vaccine applications, robust antigen-specific antibody titers and T cell responses ($CD4^+$ and $CD8^+$) were observed at a 50 μg doses of RNA after IM EP (Cu et al., 2013). These responses were statistically superior to those

elicited by naked mRNA delivery by IM injection at the same dose without EP. However, although comparable to 50 μg of DNA vaccine delivered by IM EP, the magnitude of EP versus needle inoculation enhancement of the antibody response was consistently lower for the RNA vaccine also delivered by EP. In addition, the immune responses with EP RNA were lower than those elicited by LNP delivery of self-amplifying mRNA at low doses of RNA (0.1–1.0 μg) (Geall et al., 2012). Taken together, these results suggest that EP can be effective in RNA delivery, but there is room for improvement via optimization of EP conditions of RNA. Interestingly, RNA vaccination resulted in a balanced IgG1/IgG2a response. In contrast, pDNA vaccinated groups exhibited a response skewed towards IgG1.

5.2.4 Lipid Nanoparticles

Liposomes have been explored extensively for vaccine applications (Allison & Gregoriadis, 1974; Baca-Estrada, Foldvari, Snider, van Drunen Littel-van den Hurk, & Babiuk, 1997; Demana, Fehske, White, Rades, & Hook, 2004; Giddam, Zaman, Skwarczynski, & Toth, 2012; Gregory, Titball, & Williamson, 2013; Immordino, Dosio, & Cattel, 2006). Cationic lipids, which form a variety of liposomal structures, have also been studied as gene delivery vectors for vaccine purposes (Deering et al., 2014; Gregoriadis, Bacon, Caparros-Wanderley, & McCormack, 2002; Karmali & Chaudhuri, 2007; Li & Huang, 2006; Liu & Huang, 2002; Manthorpe et al., 2005; Perrie, Frederik, & Gregoriadis, 2001; Vajdy et al., 2004). However, development of the technology has been hampered by the complexity of the formulation process and issues with large-scale manufacture. A number of methods have been tested to encapsulate nucleic acids in liposomes, including: reverse-phase evaporation, ether injection, detergent dialysis, lipid hydration, dehydration-rehydration-extrusion, freeze thaw extrusion, ethanol destabilization, ethanol Ca^{2+} destabilization and ethanol dilution. Of these methods, ethanol dilution has been shown to be the most versatile for formulation screening, scale-up and manufacturing for pre-clinical and clinical study material (Ambegia et al., 2005; Geall et al., 2012; Jeffs et al., 2005; Maurer et al., 2001; Morrissey et al., 2005; Zimmermann et al., 2006). The utility of ethanol dilution has been demonstrated for the intravenous administration of RNAi-based therapeutics with LNPs containing ionizable amino lipids, and there are currently several LNP-based siRNA formulations in various stages of clinical development (Tam, Chen, & Cullis, 2013).

We have explored ethanol dilution to encapsulate self-amplifying mRNA in liposomes or LNPs (Geall et al., 2012; Hekele et al., 2013) due to its amenability to large-scale production. For the initial proof-of-concept studies for LNP-facilitated delivery of self-amplifying mRNA, we used the stable nucleic acid lipid particle (SNALP) concept previously described by Jeffs et al. (2005). The ionizable cationic lipid 1, 2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) (Heyes, Palmer, Bremner, & MacLachlan, 2005) is highly effective in delivering siRNA systemically in rodents and NHPs. In our experiments, the additional lipid components were 1, 2-diastearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, (chol), and 1, 2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG DMG 2000) (Figure 3). The molar percent ratios of lipid components in the LNP formulation were 10:48:2:40 (DSPC: cholesterol: PEG-DMG 2000:

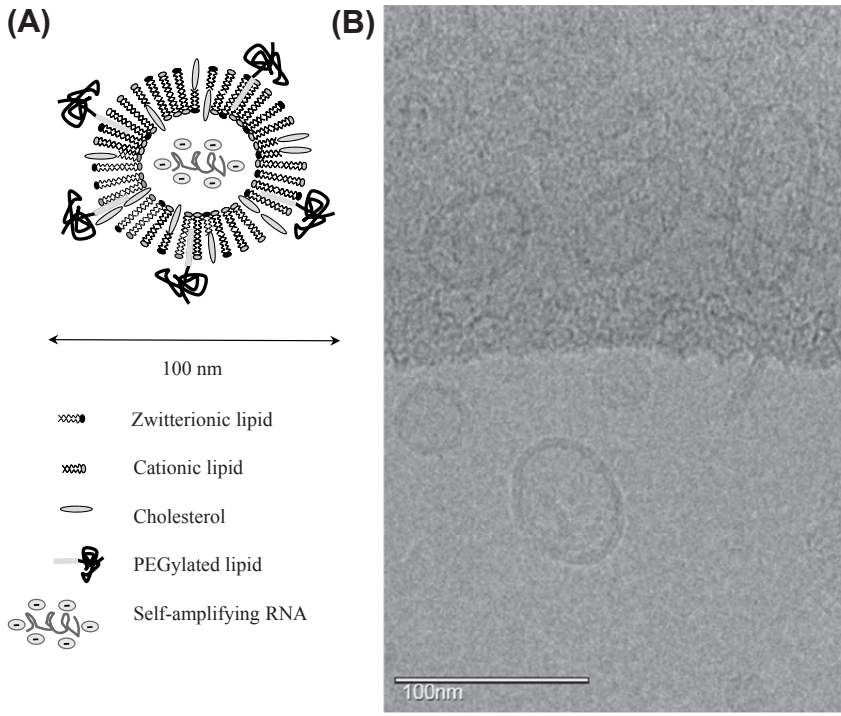

Figure 3 Lipid nanoparticle (LNP) structure. (A) Schematic representation of a LNP composed of zwitterionic lipid, cationic lipid, cholesterol, and PEGylated lipid, formulated with self-amplifying mRNA. (B) Cryotransmission electron microscopy of LNPs formulated with self-amplifying mRNA.

DLinDMA) and a 8:1 N:P molar ratio (nitrogen on DlinDMA to phosphate on RNA) (Geall et al., 2012; Jeffs et al., 2005).

Cationic lipids such as DlinDMA are weak bases with an apparent acid dissociation constant (pKa) of 6.7 (Heyes et al., 2005). Therefore, the degree to which they are protonated, or positively charged in solution, is highly dependent on the pH of the aqueous environment. During formulation, the pH of the aqueous buffer system (RNA feed stream) was lowered to pH 6 with a citrate buffer, which results in protonation of the cationic lipid upon mixing in the ethanol feed stream containing the lipid. The positive charge on the cationic lipid allows a charge: charge interactions with the phosphate backbone of the self-amplifying mRNA, which results in efficient encapsulation in the LNPs. The ethanol dilution process for LNP encapsulation of self-amplifying mRNA is described in Figure 4.

In combination with the charge: charge interactions of the cationic lipid and RNA phosphate, the mixing of the RNA-buffer and lipid-ethanol flow streams results in instantaneous dilution of ethanol below the concentration required to support lipid solubility. This driving force aids in the production

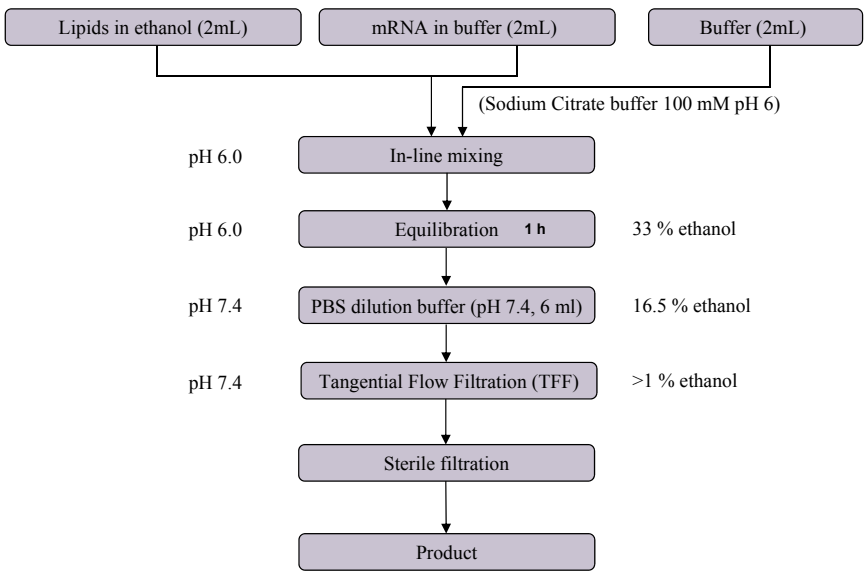

Figure 4 Ethanol dilution process for preparation of lipid nanoparticles (LNPs). Lipids are dissolved in ethanol and mixed with RNA in a buffer at pH 6.0. The resulting solution is equilibrated at pH 6.0 for 1 h, is diluted in PBS to increase the pH to 7.4 and undergoes tangential flow filtration for buffer exchange. The LNPs are then passed through a 0.22 μm filter and filled into vials.

of a monodisperse lipid particle with a high RNA encapsulation efficiency. The ethanol is then removed, and the buffer exchanged to PBS using tangential flow filtration at a large scale or dialysis at a small scale. PBS has a neutral pH (7.4), which imparts a net neutral surface charge to the LNP. More extensive evaluations of LNP siRNA systems have been conducted by others (Crawford et al., 2011; Leung et al., 2012; Semple et al., 2001; Tam et al., 2013). These experiments have indicated that LNPs have an interior lipid core containing siRNA complexed with ionizable cationic lipids. Computer simulations of the self-assembly of lipids suggest a nanostructure core, in which the siRNA is located in internal inverted micelles complexed with ionizable lipids (Tam et al., 2013). Furthermore, according to the models, DSPC interacts with the siRNA phosphate through its choline group, cholesterol is dispersed evenly between the core of the lipid particle and surface, and the PEG-lipid is distributed predominantly on the surface. Preliminary electron cryomicroscopy (cryo-EM) studies of LNP-encapsulated self-amplifying mRNA have indicated that the ethanol dilution process forms a lipid structure around an aqueous core (Figure 3). More extensive evaluations should reveal the details of the structures produced.

The PEG-lipid prevents aggregation during the formulation process, and was originally designed to provide "stealth-"like characteristics post intravenous administration for siRNA delivery applications (Tam et al., 2013). The role of the PEG-lipids in intramuscular vaccine applications has not yet been determined; but they may stabilize the LNP during formulation. Ethanol dilution produces small uniform lipid particles with a high RNA encapsulation efficiency (Geall et al., 2012). Using agarose gel electrophoresis we have shown that RNA integrity was maintained during formulation and the LNP protects the RNA from degradation by RNase A (Figure 5). In contrast, naked control RNA was susceptible to degradation by RNase A (lane 3 compared to lane 2).

After *in vivo* administration, the LNP-RNA complex will be exposed to the pH of the local environment near the injection site. Typically, physiological pH is approximately 7.4, at which very few of the ionizable cationic lipid molecules will carry a positive charge, and the overall surface charge on the liposome will be close to neutral. In contrast, liposomes that contain a cationic lipid with a quaternary amine will have a positive surface charge. Ionizable cationic lipids with a pKa of seven or lower have been shown as critical to the encapsulation of nucleic acids and delivery *in vivo* (Maurer et al., 2001; Semple et al., 2010, 2001; Tam et al., 2013). Efficient delivery

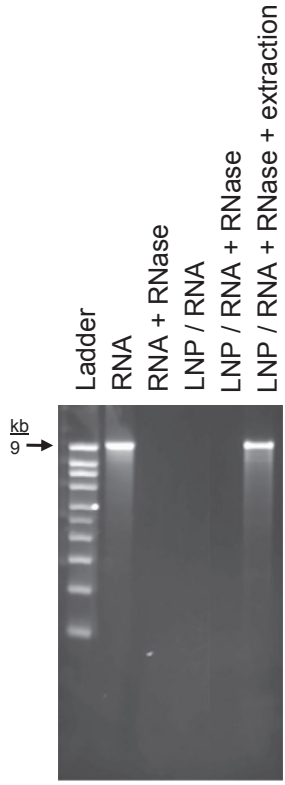

Figure 5 Agarose gel electrophoresis of an RNA molecular ladder, self-amplifying mRNA, self-amplifying mRNA incubated with RNAse, self-amplifying mRNA encapsulated in a lipid nanoparticle (LNP), self-amplifying mRNA encapsulated in an LNP treated with RNase, and self-amplifying mRNA encapsulated in an LNP treated with RNase, then extracted after RNase inactivation.

of nucleic acids to the cytosol of the target cell can be facilitated by the acidic environment of endosomes, where the cationic lipid becomes protonated, associates with the anionic endosomal lipids and enables destabilization of the endosomal membrane, thereby promoting release of the nucleic acid into the cytosol (Hafez, Maurer, & Cullis, 2001; Xu & Szoka, 1996).

The means of delivery of lipid-complexed self-amplifying RNA vaccines and their fate inside target cells remains to be determined. Self-amplifying mRNA-specific imaging experiments, as well as a more detailed *in vivo* tracking of the mRNA self-amplification process and kinetics of antigenic gene expression are required to better understand the activity of this novel nucleic acid cargo. Imaging studies to track fluorescently-labeled siRNA

delivery by LNPs has revealed that complexed RNA enters the cell through receptor-mediated endocytosis and is deposited into early endosomal vesicles, though the intracellular interactions of the payload still need to be further elucidated (Jarve et al., 2007; Rejman, Bragonzi, & Conese, 2005; Ruthardt, Lamb, & Brauchle, 2011). Application of these methods in mRNA vaccines will determine whether the route of RNA uptake is generic or dependent on the nature or the RNA or formulation.

Previously, we demonstrated in preclinical animal models that LNP delivery of self-amplifying mRNA vaccines elicits antigen-specific humoral and cellular immune responses (Cu et al., 2013; Geall et al., 2012; Hekele et al., 2013). In mice, LNP delivery resulted in higher levels of reporter gene expression (secreted embryonic alkaline phosphatase (SEAP) and luciferase) than naked RNA delivery and considerably lower variability of expression between animals (Figure 6) (Geall et al., 2012). These effects are likely achieved by a combination of increased transfection efficiency and protection of the RNA from enzymatic degradation at the site of injection.

We have performed animal immunogenicity studies using LNP-delivered self-amplifying RNA vaccines encoding respiratory syncytial virus (RSV), influenza and human immunodeficiency virus (HIV) antigens (Geall et al., 2012; Hekele et al., 2013). LNP delivery of a self-amplifying RNA encoding the fusion (F) protein of RSV elicited potent and protective immune responses comparable to those elicited by viral delivery technology and without the inherent limitations of viral vectors (Geall et al., 2012). LNP delivery of mRNA at a 0.1 μg dose of RNA

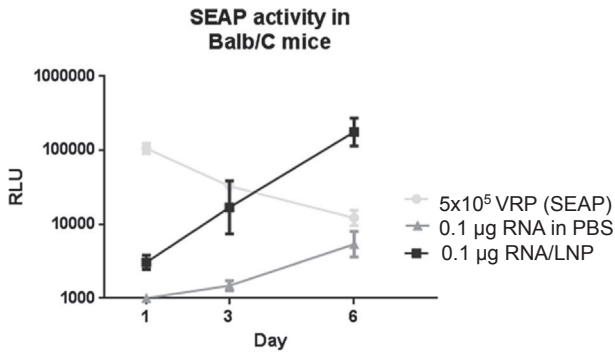

Figure 6 Secreted embryonic alkaline phosphatase (SEAP) activity in the serum of BALB/c mice (n = 5/group) expressed as relative light units 1, 3, and 6 days after intramuscular inoculation with 5×10^5 VRP, 0.1 μg self-amplifying mRNA in PBS, or 0.1 μg self-amplifying mRNA encapsulated in LNPs.

(RNA/LNP) was significantly more immunogenic than a 10-fold higher dose of naked mRNA (1 μg), and seroconversion was established after a single vaccination with doses as low as 0.01 μg. In mice, LNP delivery of self-amplifying mRNA elicited a T_H1 helper T cell phenotype, with slightly elevated titers of F-specific IgG2a relative to IgG1. In addition and consistent with this phenotype, the LNP/RNA potently elicited antigen-specific interferon-γ producing $CD4^+$ and $CD8^+$ T cell responses. The LNP/RNA matched the efficacy of a VRP delivery technology in protection against intranasal RSV challenge of cotton rats. LNP delivery of a self-amplifying mRNA encoding an HIV envelope protein gene (Env, SF162 gp140) elicited robust antibody and T cell responses after one or two intramuscular immunizations of mice (Geall et al., 2012). This vaccine candidate was also immunogenic after the intradermal and subcutaneous injection, though less immunogenic than after the intramuscular injection.

We have also tested this platform technology in rabies and CMV immunization. The rationale for the design of these vaccine candidates and preliminary results from testing in mice are presented below.

5.2.4.1 LNP Delivery of a Rabies Self-Amplifying mRNA

The rabies virus is an enveloped, single stranded, negative-sense RNA virus of the *Lyssavirus* genus, zoonotic pathogens within the family *Rhabdoviridae*. With an estimated annual global human mortality of about 24,000 to 90,000 (Knobel et al., 2005), rabies has been identified as one of the major causes of death from infectious diseases in humans. More than 99% of these deaths occur in developing countries of Asia and Africa (Evans, Horton, Easton, Fooks, & Banyard, 2012), with infection being acquired from transcutaneous or mucosal exposure to saliva of a rabid animal. Rabies manifests as a progressive, fatal encephalomyelitis. The rabies virus has a relatively simple RNA genome that encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). Of these proteins, G is the only target for neutralizing antibodies, which provide full protection against virus challenge (Ertl, 2009). Efficacious rabies vaccines for humans are commercially available (Warrell, 2012), and these include three killed virus tissue culture vaccines licensed for use in the developed world: human diploid cell vaccine (Imovax® Rabies, licensed in the UK), purified chick embryo cell vaccine (Rabipur®/RavAvert®, licensed in the USA) and a purified vero cell vaccine (Verorab®, licensed in Europe). Some of these vaccines are available

in the developing world, and less expensive alternatives are also produced using older production methods, in which the rabies virus is grown in sheep, goat or suckling mouse brains, which are homogenized before the virus is inactivated. Rabies vaccines for pre-exposure prophylaxis must be given three times to achieve protective immunity, which can last 3–4 years. For post-exposure prophylaxis of individuals exposed to rabies, the vaccines have to be given 4–5 times, and in cases of severe exposure, they have to be combined with rabies virus-specific immunoglobulin (RIG).

There is still a need for new and improved vaccines to reduce the toll of rabies disease in the developing world. These vaccines need to be inexpensive, safe, and able to provide sustained protection, preferably after a single administration. Several approaches are in preclinical development. These include attenuated viral, DNA, protein subunit and recombinant viral vector vaccines (Ertl, 2009; Hicks, Fooks, & Johnson, 2012). DNA vaccines have been proposed as a less expensive and more efficient strategy for rabies prophylaxis in humans, and their feasibility has been demonstrated in multiple preclinical models, including: mice (Lodmell, Ray, & Ewalt, 1998; Margalith & Vilalta, 2006; Ray, Ewalt, & Lodmell, 1997), rabbits (Margalith & Vilalta, 2006), horses (Fischer, Minke, Dufay, Baudu, & Audonnet, 2003), dogs (Gupta et al., 2009) and NHPs (Lodmell, Parnell, Bailey, Ewalt, & Hanlon, 2002; Lodmell, Ray, Parnell, et al., 1998). However, despite proven preclinical efficacy, DNA vaccines have considerable drawbacks to overcome. These include high doses and weaker and delayed immune responses in larger animals compared to the existing commercial vaccines.

More recently, "naked" delivery of a self-amplifying mRNA vaccine has shown potential as a prophylactic rabies vaccine (Saxena et al., 2009). We chose to make a self-amplifying mRNA rabies vaccine that encoded the rabies glycoprotein G antigen, unmodified from the full-length wild-type G from the Flury low egg passage (LEP) rabies virus strain in the commercial, inactivated virus rabies vaccine, Rabipur®. The antigen includes the transmembrane and cytoplasmic domains, and the nucleotide sequence was mammalian codon optimized. The self-amplifying mRNA was formulated with an LNP delivery system or a CNE (see next section for details) to produce two prototype vaccines. Female BALB/c mice, aged 8–10 weeks and weighing about 20 g, were immunized on days 0, 7, and 21 with Rabipur or on days 0, 21, and 45 with the self-amplifying mRNA vaccines. Serum samples were collected on days 0, 14, 35, 56, 90, and 180 after the first vaccination. Rabies G-specific IgG titers were determined by ELISA (Figure 7(A)), and rabies virus neutralizing antibody levels were determined

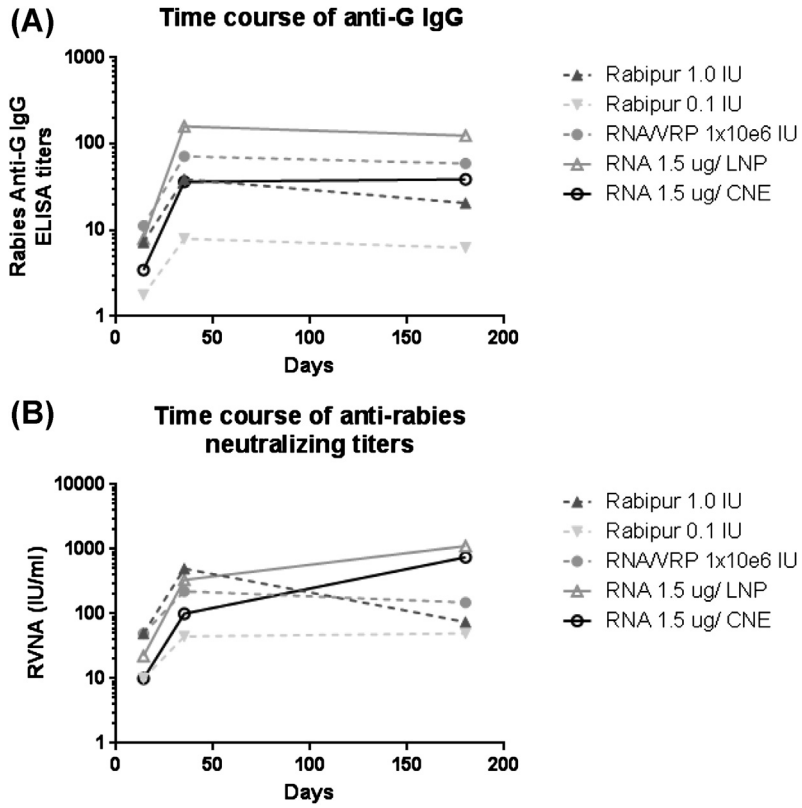

Figure 7 (A) Time course of rabies anti-G ELISA titers. (B) Rabies virus neutralization assay titers measured 14 days, 35 days, and 180 days after the first intramuscular immunization. BALB/c mice were immunized with 1 IU Rabipur, (dark gray closed upward triangle/dashed line) 0.1 IU Rabipur (light gray closed downward triangle/dashed line), on days 1, 7, and 21, or with 1×10^6 IU VRP (gray closed circle/dashed line), 1.5 μg self-amplifying mRNA formulated with LNP (RNA/LNP, open triangle/solid line), or 1.5 μg self-amplifying mRNA formulated with CNE (RNA/CNE, open circle/solid line) immunized on days 0, 21, and 42.

using a rapid fluorescent focus inhibition test (RFFIT, Figure 7(B)) on pooled samples (five/pool).

The commercial vaccine (Rabipur) showed a dose response with the higher dose, one-tenth the human clinical dose, producing the more robust responses. The LNP- and CNE-delivered RNA vaccines produced measurable rabies glycoprotein G-specific IgG titers at all-time points tested, with the 1.5 μg dose of RNA/LNP eliciting higher titers at days 35 and 180 than the high-dose commercial vaccine. The LNP and CNE-delivered RNA vaccines were at least comparable to the VRP vaccine control, which

consisted of the same RNA packaged into a viral particle delivery system and administered at a dose of 1×10^6 infectious units (IU), consistent with our previously published data in RSV and HIV (Geall et al., 2012; Hekele et al., 2013).

The LNP and CNE-delivered self-amplifying mRNA rabies vaccines produced measurable rabies virus neutralization titers at all-time points tested, with the 1.5 μg dose of RNA/LNP and RNA/CNE eliciting higher titers at day 180 than the high-dose commercial vaccine. Again, the immunogenicity of the mRNA vaccines was at least comparable to the VRP control. In mice, neutralization titers ranging from 1:5 to 1:40 are considered to be predictive of protection in the challenge model. Therefore, the titers and kinetics measured after the second vaccination are well above this protective threshold (Welch, Anderson, & Litwin, 2009). Further research will be required to evaluate this vaccine, particularly in larger animals with a pathogenic challenge.

5.2.4.2 LNP Delivery of a Human Cytomegalovirus Self-Amplifying mRNA

Human cytomegalovirus (HCMV) is an enveloped, double-stranded DNA virus and is a member of the betaherpesvirus family. The genome is 240,000 base pairs, with estimates of 165–252 ORFs (Chee et al., 1990; Dolan et al., 2004; Stern-Ginossar et al., 2012). The virus establishes a lifelong infection in humans, characterized by periods of latency and sporadic viral replication. In the United States, CMV seroprevalence increases gradually with age, from 36% in 6–11 year olds, to 49% in 20–29 year olds, and to 91% in those aged 80 years or older (Staras et al., 2006). For the vast majority of individuals, CMV infection is asymptomatic and does not pose any serious health problem. However, CMV is an important pathogen for individuals who become immunocompromised, for example, during solid organ and hematopoietic cell transplant. Despite active monitoring and antiviral therapy, the incidence of CMV infection in these patients is high, ranging from 20% to 70% in the first-year posttransplantation, and antiviral use is more practical for preventing early posttransplant infections than late posttransplant infections (Wang & Fu, 2014). Infection can occur as a newly acquired infection, in seronegative recipients receiving a transplant from a seropositive donor, or due to reactivation of latent virus or a superinfection in seropositive recipients. There is therefore an unmet medical need for a vaccine to prevent CMV infection or reactivation post transplantation (Krause et al., 2013; Wang & Fu, 2014). CMV is also estimated to cause congenital infection in 0.5–2% of all pregnancies each year in developed countries (Kenneson

& Cannon, 2007) and can lead to debilitating effects such as mental retardation, hearing or vision loss, cerebral palsy, and mortality. The Institute of Medicine has identified the development of an effective CMV vaccine for the prevention of congenital CMV infection as a top priority (Arvin, Fast, Myers, Plotkin, & Rabinovich, 2004).

There is a long history of the use of live attenuated viruses and viral vectors to develop HCMV vaccine candidates, but these have been met with limited success (Schleiss & Heineman, 2005). Several vaccine candidates are currently under clinical evaluation and these include a VRP (Bernstein et al., 2009), pDNA (Wloch et al., 2008), adjuvanted protein subunit (Frey et al., 1999; Pass et al., 1999), and peptide vaccines (La Rosa et al., 2012). (For a review see (Dasari, Smith, & Khanna, 2013; McVoy, 2013; Wang & Fu, 2014). Recent advances in understanding the immunology, pathology, and molecular biology of CMV suggest that an ideal CMV vaccine candidate must elicit humoral and cellular immune responses (Dasari et al., 2013). In addition to more well-established targets of neutralization, such as the glycoprotein gB, new antigens composed of glycoprotein complexes, critical for virus entry into cells have been identified. These include the gM/gN complex, the gH/gL complex, and a pentameric gH/gL/UL128/UL130/UL131A complex, reviewed in (Lilja & Mason, 2012). Recent experiments demonstrate that VRP delivery of a self-amplifying mRNA encoding the gH/gL complex (Loomis et al., 2013) and a pentameric gH/gL/UL128/UL130/UL131A complex (Wen et al., 2014) elicits broadly neutralizing immune responses against HCMV. Such constructs, could have utility in future vaccine strategies.

As a proof of concept, we made a self-amplifying mRNA HCMV vaccine that encodes the gH/gL glycoprotein complex, unmodified from the full-length wild type glycoproteins from the Merlin strain HCMV using a bicistronic vector that contained a second subgenomic promoter to express the gL gene (Loomis et al., 2013). The gH antigen included the transmembrane and cytoplasmic domains and the nucleotide sequence was mammalian codon optimized. The self-amplifying mRNA was formulated with an LNP delivery system. BALB/c mice were vaccinated twice (3 week interval between vaccinations) with RNA vaccine or a gHgL subunit adjuvanted with MF59. Spleens and sera were collected, 2 weeks after the second vaccination.

CMV gHgL-specific $CD4^+CD8^-$ and $CD8^+CD4^-$ T cells were quantified by staining for intracellular cytokines followed by multicolor flow cytometry. The neutralizing antibody responses (Loomis et al., 2013) and

frequencies of antigen-specific, splenic T cells from four individual mice per vaccine are shown in Figure 8.

The HCMV self-replicating mRNA vaccine encoding gHgL induced CMV antibodies and $CD4^+$ T cells that were similar in titer (antibody) or frequency ($CD4^+$ T cells) to those induced by MF59-adjuvanted gHgL protein. Moreover, the RNA vaccine induced a high frequency of antigen-specific $CD8^+$ T cells, whereas the MF59-adjuvanted gHgL protein did not induce antigen-specific $CD8^+$ T cells. Further research will be required to evaluate this vaccine approach, particularly with the pentameric CMV antigen and other T cell antigens such as pp65 and IE1.

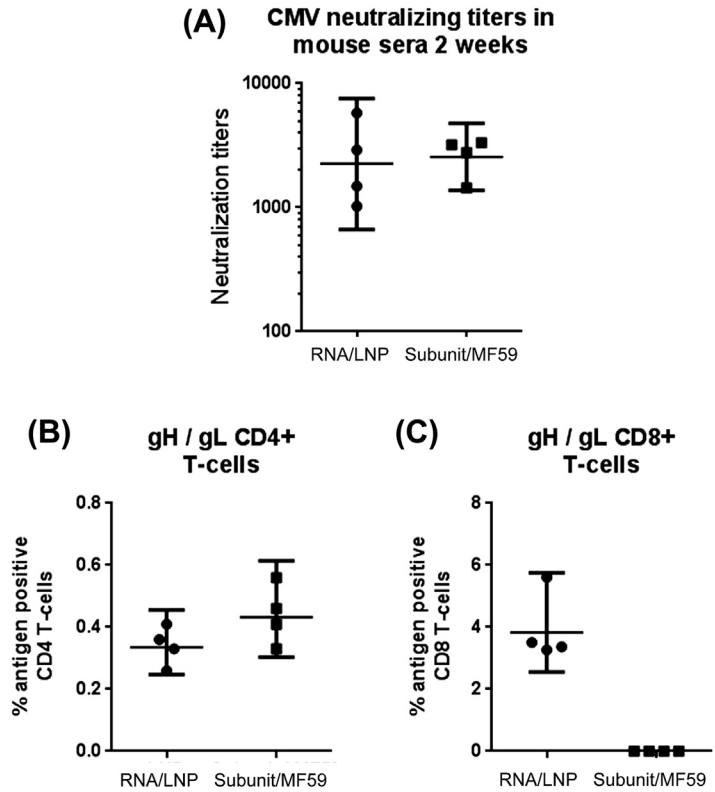

Figure 8 (A) Neutralization activity of sera against HCMV isolate VR1814 on ARPE-19 cells. (B) Splenic $CD4^+$ T cells, and (C) splenic $CD8^+$ T cells 2 weeks after two intramuscular immunizations spaced 3 weeks apart in BALB/c mice ($N = 4$/group). Mice were immunized with 1 μg of self-amplifying mRNA expressing gH/gL from HCMV (RNA/LNP, solid circles) or 1 μg gH/gL subunit protein adjuvanted with MF59 (solid squares).

5.2.5 Cationic Nanoemulsions

Emulsions have been used to increase the potency of vaccines since the use of mineral oil emulsified with mycobacteria called Freund's complete adjuvant was first introduced in 1937 (Freund, Casals, & Hosmer, 1937). This emulsion improved immune responses dramatically, but was unfortunately too reactogenic for routine clinical use due, at least in part, to poor quality of the oil. It took 60 years before a well-tolerated emulsion adjuvant (MF59®) was approved for use in a human vaccine, Fluad® for seasonal influenza for the elderly in Europe (O'Hagan, Ott, Nest, Rappuoli, & Giudice, 2013). Since the approval of Fluad®, there have been four other adjuvanted influenza vaccines containing squalene emulsions approved for use, Aflunov® (MF59 adjuvanted pandemic influenza), Focetria® (MF59 adjuvanted pandemic influenza), Prepandrix® (AS03 adjuvanted prepandemic influenza), and Pandremix® (AS03 adjuvanted pandemic influenza). In addition, squalene-based emulsions have been used in clinical trials for a range of vaccine candidates, including those targeting HSV, HIV, HCV, and CMV (De Gregorio, Tritto, & Rappuoli, 2008; O'Hagan et al., 2013).

Squalene-based emulsion adjuvants have a good record of safety and MF59-adjuvanted influenza vaccines have been shown to be effective and have a good safety profile in a wide range of patients, including infants as young as 6 months of age (O'Hagan et al., 2013). Squalene-based emulsions have also been used to deliver pDNA vaccines, where improvements in potency were observed in animal models (Ott et al., 2002). Recently, we developed an emulsion similar to MF59 and composed of squalene, Tween, and Span with the addition of the cationic lipid DOTAP for delivery of self-amplifying RNA (Brito et al., 2014). Preclinically, DOTAP has been evaluated for nonviral delivery of pDNA and has been tested clinically for delivery of pDNA in addition to the anticancer agent paclitaxel (Fasol et al., 2012; Lu et al., 2012).

When combined with nucleic acids, DOTAP liposomes typically form lipoplexes with a very complex layering of the nucleic acid cargo and cationic lipid (Koltover, Salditt, Radler, & Safinya, 1998). These mixtures can be very heterogenous, large in particle size and unstable (Eastman et al., 1997; Woodle & Scaria, 2001). In contrast, the presence of an oil core in the CNE system developed for self-amplifying mRNA leads to a homogenous, reproducible mixture. Figure 9(A) shows a schematic representation of this CNE, and Figure 9(B) a cryo EM image illustrating consistent size and morphology.

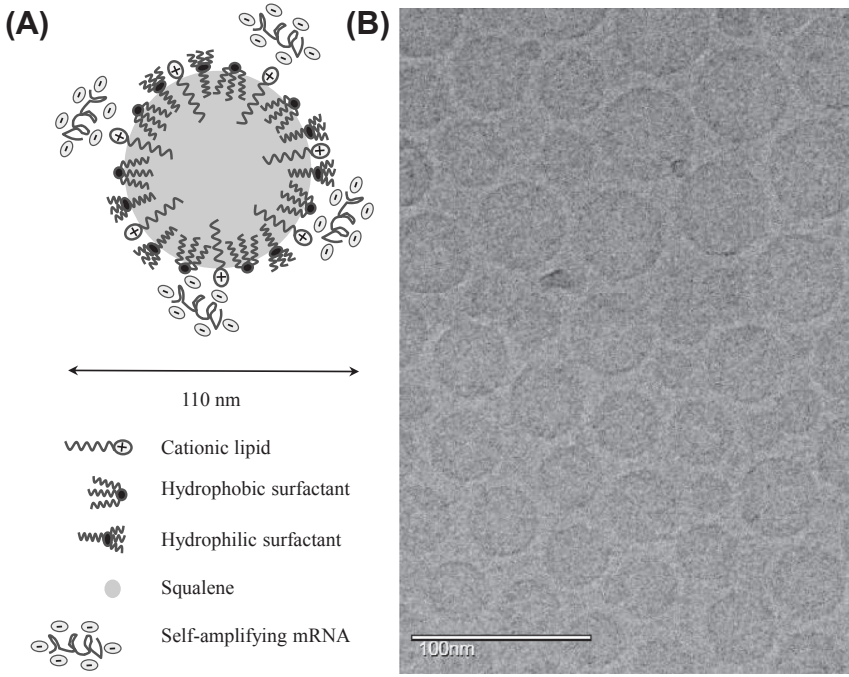

Figure 9 Cationic nanoemulsion (CNE) structure. (A) Schematic representation of a CNE composed of cationic lipid, hydrophobic surfactant, hydrophilic surfactant, and squalene formulated with self-amplifying mRNA. (B) Cryotransmission electron microscopy of CNE.

When combined with RNA, the CNE binds tightly to the nucleic acid and prevents RNAse-mediated degradation (Figure 10). Binding is reversible and the nucleic acid can be eluted from the emulsion by the addition of heparin sulfate, indicating that ionic interaction is the primary means of interaction (unpublished results). DOTAP was selected as the cationic lipid in CNE due to its ability to maintain a positive charge. The strong interaction between the positively charged lipid and the negatively charged RNA allows the delivery system and RNA to be formulated separately and mixed just before administration, with complexes forming rapidly and reliably upon mixing. As illustrated above and in Section 7, RNA can be degraded by various pathways, particularly in aqueous media. The ability to stabilize RNA without the interaction of additional components minimizes potential stability issues. Additionally, the delivery vehicle can be prepared and stored separately from the RNA drug substance for later use, as in the case of a pandemic outbreak. Preparation of CNE is similar to MF59, with the

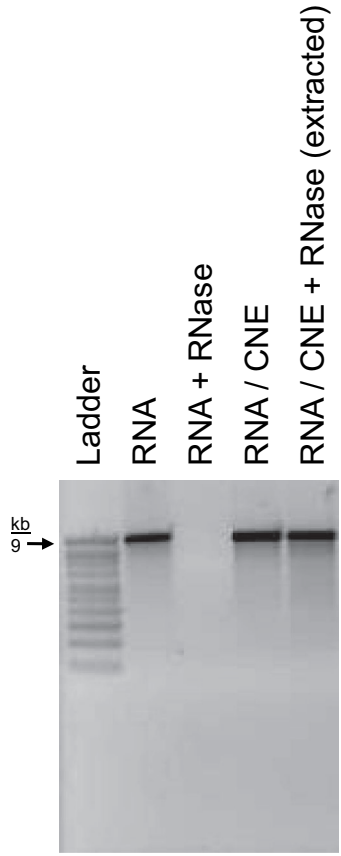

Figure 10 Agarose gel electrophoresis of an RNA molecular ladder, self-amplifying mRNA, self-amplifying mRNA incubated with RNAse, self-amplifying mRNA complexed with cationic nanoemulsion (CNE) (RNA/CNE), and self-amplifying mRNA complexed with CNE and treated with RNase, then extracted after RNase inactivation.

addition of DOTAP in the initial oil preparation step. Figure 11 illustrates the method of preparation of CNE and its mixing with RNA.

CNE is an effective mRNA delivery technology in a number of animal models (mice, rats, rabbits, and NHPs) and disease targets, including those for RSV, CMV, and HIV (Brito et al., 2014). Immunogenicity of the mRNA vaccine was found to be similar to that for a viral vector and subunit protein-based vaccines, and doses as low as 0.01 μg of self-amplifying mRNA were able to generate immune responses in mice (Brito et al., 2014). When tested in rhesus macaques, robust T cell responses and antibody responses were generated by a 50 μg dose of self-amplifying mRNA; equivalent to those elicited by a viral vector or MF59-adjuvanted

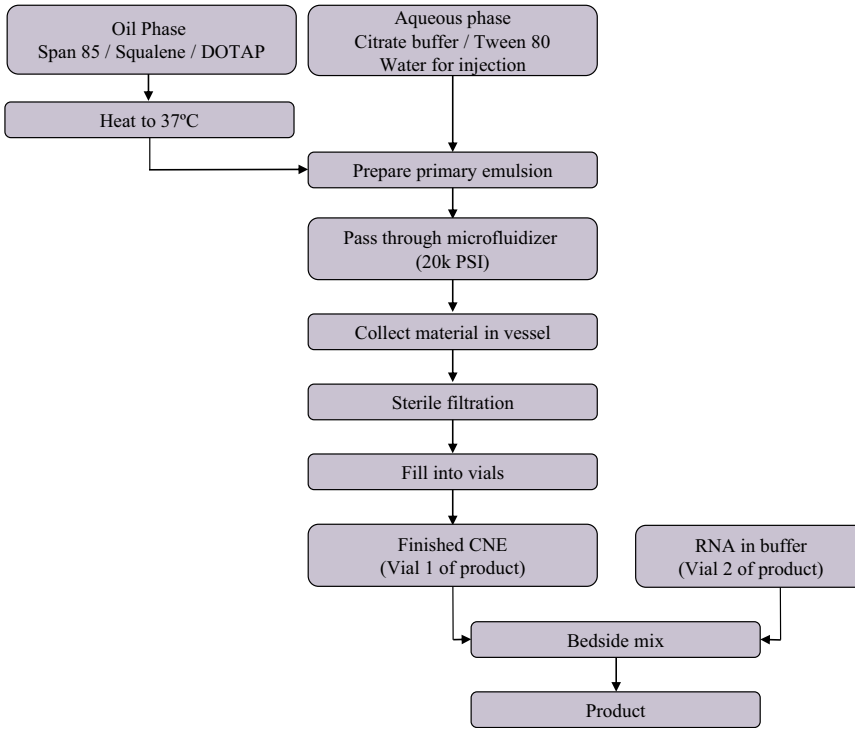

Figure 11 Preparation of cationic nanoemulsion. An oil phase containing squalene, Span 85, and DOTAP are heated to 37 °C and mixed with an aqueous phase containing a citrate buffer and Tween 80 to prepare a coarse emulsion to provide a homogenous stock solution. The coarse emulsion is passed through a microfluidizer to further reduce the particle size. Material is collected, passed through a 0.22 μm filter, and filled into vials.

subunit protein at doses previously tested in human clinical trials (Bogers et al., 2014). These encouraging responses illustrate the potential of this system.

The reporter protein luciferase was used to better understand the protein expression profile of self-amplifying mRNA delivered by CNE in mice. Similar to the observations with LNP delivery, the duration of expression was slightly longer than with a viral vector, but returned to baseline by 45–60 days (Brito et al., 2014; Geall et al., 2012). An additional study using the reporter protein SEAP was performed to investigate the expression profile of a CNE delivered self-amplifying mRNA compared to a viral vector in rhesus macaques, and the results were comparable for both technologies.

The mechanism of action for the emulsion adjuvant MF59 has been recently described in a series of papers (Calabro et al., 2011, 2013; Mosca

et al., 2008; O'Hagan, Ott, De Gregorio, & Seubert, 2012; Seubert, Monaci, Pizza, O'Hagan, & Wack, 2008; Vono et al., 2013). MF59 improves the immune responses by creating an immunostimulatory environment at the injection site, facilitating recruitment and activation of antigen presenting cells. MF59 exerts its effects in a toll-like receptor independent manner (Calabro et al., 2011; Seubert et al., 2008), involves ATP (Vono et al., 2013), and requires formulation into a nanoemulsion, as the individual components of the emulsion do not improve immune responses (Calabro et al., 2013). Although our understanding of CNE is limited so far, insight is being gained. Similar to MF59, CNE creates an immune competent environment, with a cellular infiltrate (Brito et al., 2014). Similar to a viral vector, CNE has been shown to transfect a relatively small number of myocytes at the site of injection (Geall et al., 2012). Further studies are needed to elucidate mechanism of action, in order to take rational approaches to improving mRNA delivery.

In addition to testing the CNE for the delivery of self-amplifying mRNA vaccines encoding antigens from RSV, CMV, and HIV, we have also tested this platform technology in the context of HCV. The rational for the design of this vaccine and preliminary results from testing in mice are presented below.

5.2.5.1 CNE Delivery of a HCV Self-Amplifying mRNA

HCV is responsible for most non-A/non-B types of hepatitis in humans. The virus is often transmitted parenterally through contaminated blood products or medical devices; those particularly affected are health care workers, hemodialysis patients, and intravenous drug users. Although approximately 20% of acute infections resolve spontaneously, the remaining infected individuals develop chronic hepatitis. A significant proportion of chronic HCV infection leads to liver cirrhosis and hepatocellular carcinoma. As much as 3% of the world population, or 170 million people, are affected by chronic HCV infection. Therefore, prevention and therapy for HCV infection remain significant medical needs.

HCV is an enveloped, single stranded, positive-sense RNA virus, belonging to the family *Flaviviridae*. The genomic viral RNA is approximately 9.6 kb long, and encodes a single polyprotein. The polyprotein is processed by cellular and viral proteolytic enzymes to generate 10 mature viral proteins, including the structural (core, E1, E2, p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins. The E1 and E2 envelope glycoproteins (which are encoded in the first one-third of the viral genome), are

exposed on the virion surface. The structural HCV glycoproteins (E1/E2) are critical in attachment to host cells and internalization of the viral particles ((Pileri et al., 1998) and reviewed in (Zeisel, Felmlee, & Baumert, 2013)). In addition, the presence of antibodies blocking the binding of the E2 glycoprotein to cells is correlated to resolving acute infections (Ishii et al., 1998). Anti-E1 antibody responses also contribute to viral neutralization (Fournillier et al., 1999; Meunier et al., 2008). Furthermore, E1 must be coexpressed with E2 for the latter glycoprotein to fold properly (Brazzoli et al., 2005). These biochemical and serological observations rationalized inclusion of both E1 and E2 as vaccine antigens in preventing HCV infection.

Currently, no licensed vaccines against HCV infection are available. The main challenge in developing an efficacious HCV vaccine is overcoming genetic variation of the virus. There are six major genotypes, and a few subtypes have been identified according to the analysis of viral genomic sequences. In addition, a number of escape viruses have been isolated from hepatitis C patients within the same subtype. To overcome this diversity, development of vaccines that elicit broadly crossneutralizing antibodies is crucial. We tested the utility of a CNE delivered self-amplifying mRNA vaccine (RNA/CNE) in addressing whether expression of E1-E2 from the mRNA vector can elicit crossneutralizing antibody responses broader than those elicited by protein vaccination in mice.

Figure 12 below shows the neutralization titers of retrovirus-based pseudoparticles expressing E1-E2 of HCV for cellular entry and luciferase as a reporter gene (HCVpp) (Hsu et al., 2003). Although the mRNA vaccine expressing E1-E2 glycoprotein of HCV subtype 1a elicited lower serum neutralization titers to the homologous subtype than the protein consisting of recombinant protein E1-E2, the mRNA vaccination yielded higher cross-neutralization titers to 1b subtype than protein vaccination. In contrast, the mRNA vaccine expressing E1-E2 glycoproteins from the 1b subtype, did not demonstrate cross-neutralization of the 1a subtype. Further investigation in understanding why crossneutralization induced by the RNA E1-E2 vaccines between subtype 1a and 1b is not reciprocal is needed. However, the data obtained from the preliminary experiment are encouraging in applying the self-amplifying mRNA vaccine platform in developing an HCV vaccine.

A number of research groups have reported T cell responses to the nonstructural proteins also contribute toward protection from and resolution of HCV infection (Cooper et al., 1999; Cox et al., 2005; Diepolder et al., 1995; Folgori et al., 2006; Grakoui et al., 2003; Lechner et al., 2000). As discussed elsewhere in this chapter, self-amplifying mRNA

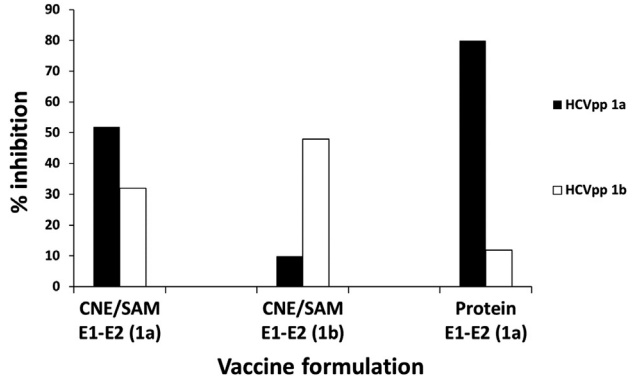

Figure 12 *In vitro* hepatitis C virus (HCV) neuralization assay measured 3 weeks after the third immunization with pooled sera. BALB/c mice (8 per group) were immunized 10 μg of self-amplifying mRNA encoding E1-E2 sequence from H77 strain (HCV genotype 1a) formulated with CNE (RNA/CNEE1-E2,1a), 10ug of self-amplifying mRNA encoding E1-E2 RNA sequence from Con1 strain (HCV genotype 1b) formulated with CNE (RNA/CNE, E1-E2,1b) and 1ug recombinant E1-E2 protein adjuvanted with MF59. Vaccines were administered on days 0, 21, and 42.

vaccines are a suitable platform in inducing robust responses from T lymphocytes, particularly Th1 and cytotoxic T cells. Preclinical studies with VRP technology with prime-boost regimens demonstrate that inclusion of nonstructural proteins in addition to the structural proteins results in elicitation of robust crossneutralizing antibodies as well as HCV-specific T cell immune responses in mice (Lin Y, 2008). Since the VRP platform is an RNA delivery system with the same viral RNA backbone used in the self-amplifying mRNA vaccine platform, it is reasonable to expect that broader crossneutralization capability may be achieved by inclusion of the nonstructural genes and/or applying prime-boost regimens with this novel nucleic acid platform.

5.2.6 Polymer PRINT™ Particles

As illustrated previously, self-amplifying mRNA can be delivered into cells using various methods. One recent approach uses insoluble proteins to encapsulate self-amplifying mRNA within particles prepared via the PRINT technology (Xu et al., 2012). This technology has been used to create particles of various sizes and shapes with exquisite control and very low polydispersity (Gratton et al., 2008). Bovine serum albumin rendered insoluble by a stimuli-responsive crosslinker was able to deliver RNA and transfect cells *in-vitro*. Follow-up studies were performed with the addition

of a lipid coating of DOTAP:DOPE in a 3:1 w/w ratio (Xu et al., 2013), which improved *in vitro* transfection.

These early results are promising, but will require confirmation in animal models. In addition, the potential of eliciting immune responses directed toward the protein components of the particle will need to be assessed for the potential to elicit antivector immunity and crossreactivity with human proteins. If so, it may be necessary to use synthetic polymers to avoid these issues.

6. PRODUCTION AND PURIFICATION OF SELF-AMPLIFYING mRNA

6.1 Cell-Free Synthesis Using an *In vitro* Transcription Reaction

Self-amplifying mRNA is produced *in vitro* using an enzymatic transcription reaction from a linear pDNA template, thereby avoiding complex manufacturing and safety issues associated with cell culture production of live viral vaccines, recombinant subunit proteins, and viral vectors. The enzymatic reaction is catalyzed by a phage RNA polymerase (Krieg & Melton, 1984; Melton et al., 1984; Milligan, Groebe, Witherell, & Uhlenbeck, 1987), and commercial *in vitro* transcription (IVT) kits that produce milligram quantities of RNA for research purposes are now available. In addition to the polymerase enzyme, IVT reactions typically include: a linearized DNA template with a promoter sequence ($\approx$23 bases) that has a high-binding affinity for its respective polymerase; ribonucleotide triphosphates (rNTPs) for the four required bases (adenine, cytosine, guanine, and uracil); a ribonuclease inhibitor to inactivate any contaminating RNase; a pyrophosphatase to degrade pyrophosphate, which will inhibit transcription; $MgCl_2$, which supplies Mg^{2+}as a cofactor for the polymerase; and a pH buffer, which also contains an antioxidant and a polyamine at the optimal concentrations (Geall et al., 2013).

The recombinant plasmid is propagated in *Escherichia coli,* linearized using a unique restriction site downstream of the transcription cassette's 3′ end, and isolated and purified using standard molecular biology techniques. During the IVT reaction, the bacteriophage polymerase binds the promoter sequence to initiates transcription, and the enzyme then moves along the template toward its 5′ end, elongating the RNA transcript as it travels. Termination of transcription occurs when the enzyme runs off of the end of the template (run-off transcription) (Figure 13).

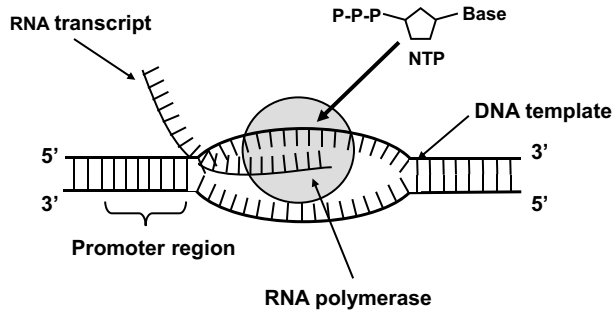

Figure 13 Enzymatic *in vitro* transcription reaction for the production of mRNA. A linearized DNA template containing a polymerase promoter sequence allows binding of the RNA polymerase, incorporation of the nucleotide triphosphates in a sequence-dependent manner as the enzyme reads from the 5′ to 3′ end and termination when the enzyme runs off the template.

The poly(A) tail can be encoded into the DNA template, or, alternatively, it can be added enzymatically posttranscription (Moon & Wilusz, 2012). When the IVT reaction is complete, the DNA template is fragmented with a DNase, and RNA is recovered using several methods, including precipitation or chromatography. The quality and quantity of RNA produced in an IVT reaction depends upon a number of factors, including RNA transcript size, template concentration, reaction time and temperature, Mg^{2+} concentration, and rNTP concentration (Davis, 1995). Typically, the conditions require some optimization for each type of construct being produced.

Most eukaryotic mRNA molecules have a 5′ 7-methyl guanosine residue-containing cap structure, which functions both in initiating protein synthesis and protecting mRNA from intracellular digestion by nucleases (Marcotrigiano, Gingras, Sonenberg, & Burley, 1997). The prevailing method used for IVT reactions employs the addition of a cap analogue as an initiator of transcription at a fourfold excess over GTP. There are two forms of the cap analogue: the pseudosymmetrical cap analogue (or Cap 0; $m^7G(5')ppp(5')G$) (Pascolo, 2004, 2008) and the antireverse cap analogues (Peng, Sharma, Singleton, & Gershon, 2002). Both add considerable costs to the IVT and, therefore, large self-amplifying mRNAs tend to be capped with an alternative, less expensive, enzymatic vaccinia virus-derived capping enzyme, which is now commercially available (Guo & Moss, 1990; Mao & Shuman, 1994). This single enzyme is composed of two subunits (D1 and D12) and has three enzymatic activities: RNA triphosphatase and guanylyltransferase by the D1 subunit, and guanine

methyltransferase by the D12 subunit. *In vitro* transcripts can be capped in less than 1 h in the presence of a capping enzyme, reaction buffer, GTP, and the methyl donor S-adenosylmethionine. Capping is nearly 100% efficient, and all capped structures are added in the proper orientation, unlike cotranscriptional addition of some cap analogs.

6.2 Purification

Large-scale RNA purification is currently a very active area of research (Breckenridge & Davis, 2000, Davis, 1995; Pascolo, 2004, 2008). The number of published patent applications in the field has recently increased, suggesting a growing interest of the pharmaceutical industry to develop platform processes for RNA manufacturing. A commercially viable purification process should be scalable, in compliance with good manufacturing practices, able to remove all the components added to the *in vitro* and capping reactions, and able to generate a sterile final bulk compatible with any requirements for formulation and *in vivo* delivery. Additionally, a process for vaccine manufacturing should avoid the use of organic solvents due to potential safety concerns regarding residual solvent, high purchase costs, environmental impact, and potential detrimental effects on RNA stability and potency.

In contrast to purification of RNA, purification of DNA for pharmaceutical applications has a long history (Cai, Rodriguez, & Hebel, 2009). GMP production is currently at the gram-to-kilogram scale, but the most commonly used production processes are very different from those required for mRNA. pDNA is produced by bacterial fermentation and the pDNA product represents about 2% (weight for weight) of the total nucleic acid in *E coli* (Ferreira, Cabral, & Prazeres, 1999). Therefore, the pDNA must be purified away from bacterial cell debris, which includes proteins, host DNA and RNA, and endotoxin. RNA is treated as a contaminant and removed with selective precipitation using Ca^{2+}, NH_4^+, or Mg^{2+} ions or polyethylene glycol (PEG) (Stadler, Lemmens, & Nyhammar, 2004). In addition, the pDNA exists in three isoforms (supercoiled, open circular, and linear) and the supercoiled species must be selectively purified above a defined specification (Cai et al., 2009; Manthorpe et al., 2005). Therefore, the downstream purification process for pDNA is complex and involves several steps: cell lysis/disruption, solid/liquid separation, isolation and purification, concentration/buffer exchange, and finally sterile filtration. A variety of chromatographic techniques have been applied to the purification of pDNA, including size-exclusion chromatography (SEC), reversed-phase

chromatography, anion-exchange chromatography, hydrophobic interaction, and thiophilic adsorption chromatography (Cai et al., 2009).

Like DNA, mRNA has a negatively charged phosphodiester backbone, and many of the purification techniques used for pDNA could potentially be adapted to the purification of this molecule. However, mRNA purification could potentially be much simpler than pDNA purification, as the components of the enzymatic transcription reaction are well defined, at relatively low concentrations, and not accompanied by host cell debris. Since the process is cell-free, regulatory requirements for viral clearance or endotoxin removal may be reduced. A possible generalized scheme for RNA production is shown in Figure 14.

The process uses recombinant or chemical products, which are of animal-free origin. Thus, contamination with viruses, retroviruses, or prion proteins is excluded. For routine preclinical work and *in vivo* immunization studies, RNA can be simply precipitated with LiCl from this reaction mixture, washed with ethanol, and resuspended in buffer for use in a vaccination. However, implementing such a process for GMP production would be extremely challenging. Historically, large quantities (milligrams) of pure RNA were required for biophysical and structural characterization, and this was achieved by denaturing polyacrylamide gel electrophoresis (PAGE) (Cunningham, Kittikamron, & Lu, 1996; Wyatt, Chastain, & Puglisi, 1991), or more recently, by SEC, which does not require denaturing conditions (Lukavsky & Puglisi, 2004). Column chromatography offers high capacity and good resolution for purifying short oligoribonucleotides, but denaturing PAGE is typically the method of choice for purifying longer (>30 mer), *in vitro* transcribed RNA. However, the loading capacity of the conventional PAGE gel is low (Cunningham et al., 1996). Recent work by Kariko, Muramatsu, Ludwig, and Weissman (2011) has

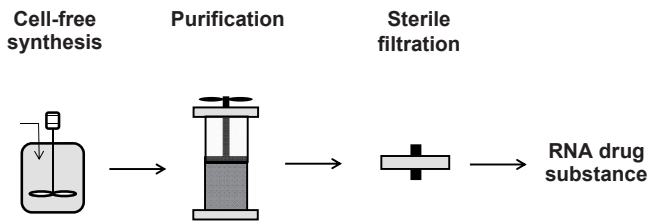

Figure 14 Schematic representation of a large-scale cell-free RNA production process. (1) Capped mRNA is produced enzymatically, and the DNA template is digested. (2) DNA fragments, transcription enzymes, reagents, and byproducts are removed using chromatographic purification. (3) RNA is then sterile filtered and stored in bulk ready for further downstream processing and formulation.

demonstrated the utility of an HPLC purification method for mRNA gene therapy applications. The method involves the use of organic solvents, and this will raise potential safety concerns about residues.

Pharmaceutical grade mRNA is currently offered by two companies: Asuragen in the United States (www.asuragen.com) and CureVac GmbH in Europe (www.curevac.com). The recent pioneering work of CureVac on the clinical production of smaller (≈2 kb) nonamplifying mRNA has established the feasibility of large-scale production of mRNA for human use in cancer vaccines (Kallen & Thess, 2014; Pascolo, 2004, 2008). Although the process remains proprietary and unpublished, CureVac has demonstrated that the chromatographic purification process separates mRNA according to size (Pascolo, 2004, 2008) and trace amounts of contaminants from the IVT reaction (proteins, DNA fragments) are removed, as are abortive (shorter) or aberrant (longer) transcripts produced during the enzymatic reaction.

Self-amplifying mRNA vaccines are larger mRNAs, with sizes in the order of 10,000 bases (MW ≈ 3 MDa). Such molecules offer additional challenges, due to size exclusion effects and poor recovery, and no commercially viable processes have yet been disclosed. Chromatographic separation of RNA based on ion-pairing reverse phase HPLC or ion exchange resins are based on the molecule's total charge. They are effective in purification of RNA molecules of up to about 4000–5000 bases, and therefore they are unlikely to find utility as purification methods for self-amplifying mRNA vaccines. Thus, there remains a need for improved RNA purification methods, and, in particular, for those that will enable cost and time efficient purification of large RNAs at an industrial scale with high yield and pharmaceutical grade purity, while retaining the stability, biological potency, and functionality of the RNA. Large-scale chromatographic purification of RNA is complex and is an active area of research for many companies and academic institutions. An RNA production process has the potential to be generic and independent of the encoded antigen in the vector, and production times could be dramatically lower than those for production of recombinant proteins.

7. STABILITY OF mRNA

There are considerable differences in stability between DNA and RNA. With over 20 years of extensive research and development of

pDNA vaccines, the major pathways of degradation in solution have been determined to be depurination/β-elimination and free radical oxidation. A rationally designed liquid formulation that is stable for 1 year at 30 °C has been developed (Evans et al., 2000). This degree of stability is unlikely in mRNA vaccines, because RNA contains a 2'hydroxyl group on the ribose, which is hydrolytically much less stable than the deoxyribose, and RNA is more sensitive than DNA to oxidation and enzymatic degradation. This section attempts to summarize what is known about mRNA stability and some of the analytical tools that might be useful in helping to characterize formulation stability.

Studies on the chemical stability of RNA, particularly on alkaline hydrolysis, started in the early 1950s (Brown, Magrath, Neilson, & Todd, 1956; Lipkin, Talbert, & Cohn, 1954). The chemical structure of RNA is shown in Figure 15(A) below. RNA has a phosphodiester bond between the phosphate backbone and ribose, and a glycosidic bond between ribose and the base. These linkages are cleavable under unfavorable conditions through hydrolysis of phosphodiester bond, hydrolysis of glycosidic bond via nucleoside modifications, and enzymatic cleavage. Although nucleoside modifications remain localized on the base, the alkaline hydrolysis and enzymatic conditions affect strand scission directly. In addition, the 5'-end of RNA has a unique m7G(5′)ppp(5′)G structure that is present in all cellular and nonorganellar RNA. An intact and stable cap structure is necessary for transcription initiation and RNA to be functional (Shatkin, 1976).

The phosphodiester bonds of RNA in aqueous solution undergo intramolecular transesterification, which involves a nucleophilic attack of the 2'-hydroxy group on the phosphate backbone, resulting in cleavage to a 2′, 3'-cyclic phosphate and isomerization to a 2′, 5'-phosphodiester. The cyclic phosphate rapidly hydrolyzes to a mixture of 2'- and 3'-phosphates (Brown et al., 1956; Mikkola, Kosonen, & Lonnberg, 2002; Oivanen, Kuusela, & Lonnberg, 1998). The presence of this hydroxyl group at the 2'-position on the ribose makes RNA a more unstable molecule and prone to strand cleavage. Therefore, it is possible that stable RNA formulations may require frozen, freeze dried, or lyophilized formulations. Preliminary published studies have indicated the feasibility of using lyophilization to stabilize RNA during storage, although much more research and development will be required to develop a commercially viable format (Jones, Drane, & Gowans, 2007; Petsch et al., 2012).

Modifications on the nucleobase by alkylation, oxidation, or electrophilic additions result in hydrolysis of glycosidic bonds creating an abasic

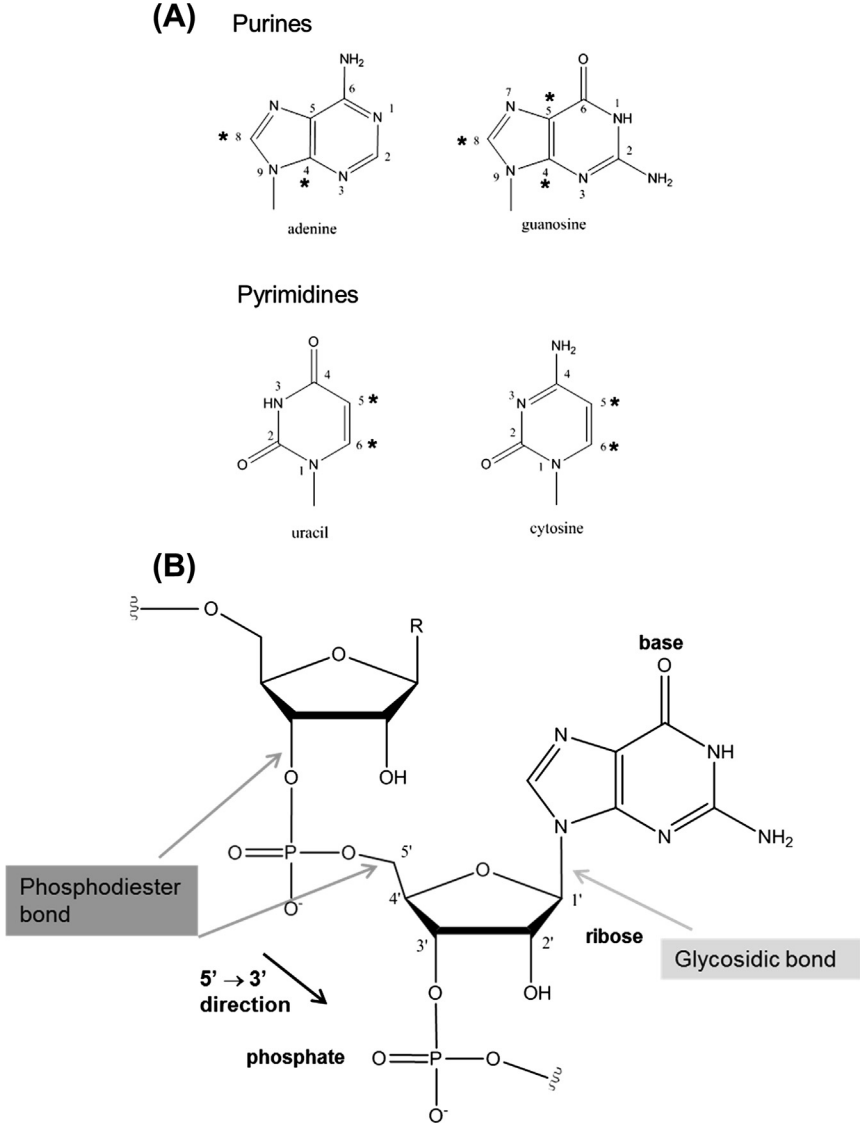

Figure 15 Chemical structure of (A) purine and pyrimidine bases present in RNA showing potential sites for oxidation (*), (B) RNA with nucleobase linked to the ribose via glycosidic bond and ribose connected to the phosphate backbone via phosphodiester bond.

site (Burrows & Muller, 1998). Figure 15(B) below shows potential sites for oxidation of nucleobases of RNA. Guanine is the most commonly oxidized purine. Guanines located 5′ to a purine, especially another G (5′-GG-3′), are more susceptible to oxidation than those next to a 3′ pyrimidine. The observed reactivity of G sequences is: GGGG > GGG > GG > GA > GT ~ GC (Kovalsky, Panyutin, & Budowsky, 1990; Muller, Hickerson, Perez, & Burrows, 1997; Saito et al., 1995). The modifications on pyrimidines are known to occur at the 5, 6 double bond causing a more labile N-glycoscidic bond, leading to abasic site formation (Setlow, Carrier, & Bollum, 1965). Reactions with alkylating agents, halogens, and most oxidizing agents do not cause direct strand scission, but the cleavage happens in the second step involving heat, alkali, or enzymatic agents. However, oxidation through nucleobase radicals, formed as reaction intermediates upon exposure of RNA to hydroxyl radicals, is known to cause direct strand cleavage by removing hydrogen atoms from the ribose ring (Jacobs, Resendiz, & Greenberg, 2010). Cleavage through β, δ-elimination has been well studied in DNA and is known to occur in RNA at position 3′ to the abasic site at high pH (Kupfer & Leumann, 2007; Tamm, Shapiro, Lipshitz, & Chargaff, 1953). Compared to DNA, the presence of the hydroxyl group at the 2′ position of the ribose sugar makes it more labile under basic conditions, and RNA is subject to alkaline hydrolysis at a much faster rate than creation of abasic site (Burrows & Muller, 1998). Recent studies have shown that the strand cleavage at the abasic site of RNA occurs through a combination of the two mechanisms, cyclophosphate formation and β,δ-elimination (Kupfer & Leumann, 2007).

There are three major classes of RNA-degrading enzymes (ribonucleases or RNases): endonucleases (which cut RNA internally), 5′ exonucleases (which hydrolyze RNA from the 5′ end), and 3′ exonucleases (which degrade RNA from the 3′ end) (Houseley & Tollervey, 2009). The mechanism of action of RNases has been extensively studied, and the enzyme is known to catalyze transesterification at a specific site (after purines) by promoting attack of the 2′ hydroxyl group on the phosphate backbone followed by hydrolysis of the cyclic phosphate diester formed in the first step. Some of these enzymatic degradations can be prevented by simply avoiding the introduction of RNase during RNA synthesis and formulation. However, during delivery, the RNA will be exposed to these enzymes in the host tissue. Recently, Geall et al. described how encapsulating a self-amplifying RNA into an LNP protected it from RNase digestion (Geall et al., 2012; Geall et al., 2013).

Exogenously delivered mRNA can also be a substrate of cellular mRNA decay pathways. Most mRNA decays in a deadenylation-dependent process in which the poly(A) tail is first removed by the deadenylases, including CCR4-NOT or PARN. Upon removal of the poly(A), the transcript is further degraded by one of two mechanisms: decapping followed by 5′ to 3′ decay, or direct 3′ to 5′ decay. These two pathways utilize specific nuclease machinery. In 5′ to 3′ degradation, the Lsm1-7 complex assembles onto the now deadenylated 3′ end of the RNA and recruits decapping enzymes, such as Dcp1 and Dcp2. Once uncapped, the RNA can be degraded by XRN1, a 5′ to 3′ exoribonuclease. In the 3′ to 5′ decay pathway, the exosome decays deadenylated RNA directly, and the remaining cap is hydrolyzed by the scavenger decapping enzyme DcpS (Figure 3) (Garneau, Wilusz, & Wilusz, 2007). Although far less common, deadenylation-independent mRNA decay pathways may also affect vaccine mRNA. By a process of endoribonucleolytic cleavage, RNA is internally broken producing two RNA fragments that can be targeted by exonucleases for degradation.

Cleavage of DNA or RNA is most commonly detected by nucleic acid fragmentation on gel electrophoresis. A number of nucleobase modifications, such as the adduct formation by oxidation, alkylation, or electrophilic addition, are not detectable using standard techniques (Kellner, Burhenne, & Helm, 2010). These modifications are in low abundance and need highly sensitive methods to profile and detect the different nucleotide variants. The most widely used techniques include thin layer chromatography, total digest analysis followed by LC-MS, primer extension, ligation, microarray analysis or capillary electrophoresis (Cornelius & Schmeiser, 2007; Dai et al., 2007; Durairaj & Limbach, 2008; Kellner et al., 2010; Motorin, Muller, Behm-Ansmant, & Branlant, 2007; Saikia, Fu, Pavon-Eternod, He, & Pan, 2010; Zhao & Yu, 2004). In addition to these techniques, reactivity toward small molecules is used for tagging and labeling modifications or abasic sites for improved detection (Sachsenmaier, Handl, Debeljak, & Waldsich, 2014). In addition to detecting nucleoside modification and strand cleavage, analytical methods to determine a stable cap structure are essential in ensuring functional RNA. Methods using a combination of the above mentioned techniques could be used to analyze the cap structure (Kuo, Smith, Shi, Agris, & Gehrke, 1986). Although most of these techniques are time-consuming, high through-put sequencing methods with high resolution are needed for sequencing long RNA. Recent technological advancements in this area have overcome limitations of the scalability of

traditional sequencing methods. Today, long RNA can be sequenced using RNA-seq or shotgun sequencing approaches that can detect single nucleotide variants or alternate splicing events that could affect expression levels (Iacobucci et al., 2012; Wang, Gerstein, & Snyder, 2009).

8. FUTURE PROSPECTS FOR NONVIRAL DELIVERY OF SELF-AMPLIFYING mRNA VACCINES

This novel vaccine technology is still in preclinical research, and the potency or efficacy of vaccines made with this technology in humans has yet to be established. However, based on the preclinical data, and like other types of nucleic acid vaccines, self-amplifying mRNA vaccines have the potential to combine the positive attributes of live attenuated vaccines while bypassing some of the inherent potential safety limitations. Large-scale production of mRNA was historically viewed as an insurmountable barrier to commercialization, but this perspective has changed, and the ease and scalability of production is now viewed as one of the greatest assets of this vaccine concept. The instabilities of mRNA must also be overcome, and this will be a key area of research in the future to enable a licensed clinical product. These barriers have in part been removed by ongoing clinical testing of small conventional mRNA cancer vaccines. Outside the vaccine field, there has been significant impetus over the past 10 years to improve the nonviral delivery of short nucleic acids (e.g., siRNA) into the cytoplasm. This has expanded our understanding of the requirements for cytoplasmic delivery of RNA, which are, less arduous than nuclear delivery of pDNA. All of these changes have spurred innovations in vector design, nonviral delivery, and large-scale production of self-amplifying mRNA to quickly move the technology forward.

While self-amplifying mRNA vaccines have medical potential, a key predictor of success in humans may be their potency and immunogenicity in larger animal species, such as NHPs. Historical data with pDNA have shown potency in larger species to be a stumbling block, with 1000-fold higher doses of DNA (mg vs μg) being required to elicit an effective immune response over those required in small animals models. Significant advancements in pDNA vaccines have been made through optimization of DNA constructs, coexpression of immune-stimulatory molecules, and improved delivery technologies. Ongoing pivotal clinical trials will ultimately determine whether these improvements are sufficient in translation into practical human vaccines. The self-amplifying mRNA vector has

been tested in humans using viral delivery and shown to be immunogenic. A live attenuated virus vaccine was developed in 1961 against Venezuelan equine encephalitis (TC-83) and has been used for vaccination of laboratory personnel at risk of occupational exposure (Weaver, Ferro, Barrera, Boshell, & Navarro, 2004). A VRP vaccine encoding CMV antigen was immunogenic in CMV seronegative individuals (Bernstein et al., 2009). Although these vaccines used viral delivery systems, they served as a proof-of-concept that the self-amplifying vector can function in humans. During preclinical testing in small animal models, self-amplifying mRNA vaccines have been benchmarked against VRP delivery and shown to produce equivalent responses, albeit at substantially increased doses of RNA. This decreased potency relative to VRP likely reflects the poor efficiency of the nonviral relative to viral delivery. In addition, experiments in small animal models have required relatively low doses of RNA when compared to pDNA vaccines, expressing the same antigen and delivered using electroporation. The initial experiment in NHPs with the CNE delivery system is very encouraging: A 50 μg dose of self-amplifying mRNA was immunogenic and elicited comparable responses to those achieved with a VRP delivery of the same RNA (Bogers et al., 2014). These data contrast with the barrier that DNA vaccines faced on the transition from small to large animal species.

The initial preclinical studies with self-amplifying mRNA vaccines for several disease targets indicate that, like pDNA, this technology has the potential to be a platform technology that could address some of the health challenges of the twenty-first century. Self-amplifying mRNA vaccines have three clear advantages over pDNA vaccines, which are as follows:

1. Self-amplifying mRNA need only to be delivered into the host cell cytoplasm that is to be translated, whereas DNA must be transported across the nuclear membrane and transcribed, a process known to be inefficient.
2. The safety concerns surrounding DNA integration into the host genome post transfection, albeit with low probability, are obviated by mRNA, as integration of RNA is not possible.
3. During self-amplification the mRNA will mimic a viral infection and potentiate an immune response against the expressed viral antigen.

An important area of research for self-amplifying mRNA vaccines is nonviral delivery, which was intensely studied in the context of DNA vaccines. Preliminary research in this area indicates that significant modifications and alternative strategies will be required for efficient delivery of mRNA (Bettinger, Carlisle, Read, Ogris, & Seymour, 2001; Huth et al., 2006;

Schlake et al., 2012; Wang et al., 2013). Initial preclinical testing has shown nonviral delivery of self-amplifying mRNA vaccines capable of producing potent and robust innate and adaptive immune responses. If these vaccines prove to be potent, immunogenic, and well tolerated in humans, we could be at the start of a new revolution in nucleic acid vaccines.

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CHAPTER EIGHT

Gene Electrotransfer Clinical Trials

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Contents

Abstract

Plasmid or non-viral gene therapy offers an alternative to classic viral gene delivery that negates the need for a biological vector. In this case, delivery is enhanced by a variety of approaches including lipid or polymer conjugation, particle-mediated delivery, hydrodynamic delivery, ultrasound or electroporation. Electroporation was originally used as a laboratory tool to deliver DNA to bacterial and mammalian cells in culture. Electrode development allowed this technique to be modified for *in vivo* use. After preclinical therapeutic studies, clinical delivery of cell impermeant chemotherapeutic agents progressed to clinical delivery of plasmid DNA.

One huge benefit of this delivery technique is its malleability. The pulse protocol used for plasmid delivery can be fine-tuned to control the levels and duration of subsequent transgene expression. This fine-tuning allows transgene expression to be tailored to each therapeutic application. Effective and appropriate expression induces the desired clinical response that is a critical component for any gene therapy.

This chapter focuses on clinical trials using *in vivo* electroporation or electrotransfer as a plasmid delivery method. The first clinical trial was initiated in 2004, and now more than fifty trials use electric fields for gene delivery. Safety and tolerability has been

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demonstrated by several groups, and early clinical efficacy results are promising in both cancer therapeutic and infectious disease vaccine applications.

1. INTRODUCTION

Gene therapy uses nucleic acids with therapeutic potential rather than drugs to treat disease. This chapter focuses specifically on the delivery of DNA encoding therapeutic transgenes. As such, the challenge is to efficiently and effectively deliver DNA to the target cells with the least amount of cellular damage and the appropriate levels and kinetics of expression. Effective delivery, producing the appropriate expression of the transgene and inducing the desired clinical response, is a critical component in achieving successful gene therapy. Different delivery approaches may be optimal for different therapeutic situations. Therefore, it is important to consider the advantages and disadvantages of the specific gene transfer technique when designing a delivery protocol to achieve a specific therapeutic response. For example, viral delivery is the chief delivery method for long-term gene expression such as in gene replacement (Kaufmann, Buning, Galy, Schambach, & Grez, 2013; Vannucci, Lai, Chiuppesi, Ceccherini-Nelli, & Pistello, 2013; Wirth, Parker, & Yla-Herttuala, 2013). However, short-term expression may be optimal when dealing with DNA vaccines or immunotherapy agents such as cytokines and it may be more appropriate for these approaches to use plasmid DNA (Keane-Myers & Bell, 2014; Lucas, Heller, Coppola, & Heller, 2002; Lucas & Heller, 2003; Heller & Heller, 2006; Heller & Heller, 2010).

Gene transfer using plasmid DNA is an attractive option because it removes the need for a biological vector, improving the safety profile compared to viral vectors (Calcedo & Wilson, 2013; Mingozzi & High, 2013; Schambach, Zychlinski, Ehrnstroem, & Baum, 2013). A disadvantage of plasmid DNA-based gene delivery is inefficient delivery across cell membranes and low levels of expression in several tissues. Delivery and subsequent expression can be enhanced to varying degrees using a variety of approaches including lipid or polymer conjugation, particle-mediated delivery, hydrodynamic delivery, ultrasound, or electroporation (Bonamassa, Hai, & Liu, 2011; Hart, 2010; Kawakami, Higuchi, & Hashida, 2008; Lavigne & Gorecki, 2006; Niidome & Huang, 2002; Noble et al., 2013; Song, Shen, Chen, Brayman, & Miao, 2011; Suda & Liu, 2007; Wang, Li, Ma, & Steinhoff, 2013).

A large number of studies have been conducted using *in vivo* electroporation demonstrating its reliability as a physical method of plasmid DNA delivery to cells. *In vivo* electroporation was first utilized to effectively deliver

chemotherapeutic agents to solid tumors in animals and humans. Clinical trials have been performed to test this therapy on multiple tumor types including melanoma, squamous cell carcinoma, liver metastases, and basal cell carcinoma (Belehradek et al., 1993; Cadossi, Ronchetti, & Cadossi, 2014; Heller et al., 1998; Matthiessen et al., 2011). The success of *in vivo* electroporation in effective and safe delivery of chemotherapeutics achieving significant local objective response rates (>70%) combined with the demonstration that electroporation could deliver plasmid DNA to cells *in vitro* provided the basis for utilizing this technique to deliver plasmid DNA *in vivo*. The use of *in vivo* electroporation to deliver DNA has also been referred to as gene electroinjection, gene electrotransfer (GET), electrically mediated gene transfer, electropermeabilization, or electrogene transfer. For the purposes of this chapter, the terms *in vivo* GET or electrotransfer will be used. The first uses of this concept were in skin, the liver and orthotopic brain tumors (Heller et al., 1996; Nishi et al., 1996; Titomirov, Sukharev, & Kistanova, 1991). Since then, this technique has been applied to many other tissues and animal models including skeletal muscle, kidney, testes, brain, cartilage, arteries, prostate, cornea, heart, and many tumor types in rodents, guinea pigs, dogs, rabbits, and nonhuman primates (Andre & Mir, 2010; Bodles-Brakhop, Heller, & Draghia-Akli, 2009; Gothelf & Gehl, 2010; Heller & Heller, 2006). This chapter will focus on the clinical translation of electrotransfer to deliver plasmid DNA. The ClinicalTrials.gov identifier is noted for registered studies with the US National Institutes of Health.

2. GET IN CLINICAL TRIALS

2.1 Overview

GET is increasingly used as a nonviral gene delivery method due to its high efficiency and relatively low adverse effects. Although it effectively delivers plasmids to a wide variety of tissue in preclinical animal studies, translation of GET applications have thus far focused on muscle, skin, and directly to tumors. Using the search terms "electroporation" or "electropermeabilization" on the ClinicalTrials.gov Web site (www.clinicaltrials.gov), an extensive database of active clinical trials located in over 170 countries, revealed 85 clinical trials currently registered in the database. Of these studies, 28 use electrochemotherapy (drug delivery) or irreversible electroporation (direct ablation of tumors). These 28 nongene transfer studies are all focused on local destruction of solid tumors. Eleven of these studies

test the efficacy of the delivery of a chemotherapeutic drug such as bleomycin delivery directly to tumors to treat head and neck cancer, colorectal cancer, cutaneous malignancies, pancreatic cancer, brain metastases, keloids, and breast cancer. Two of the studies test the efficacy of delivering calcium. The other 15 nongene transfer studies utilize irreversible electroporation to ablate the tumor and are evaluating its use in liver, pancreatic, prostate, and kidney cancers. Of the remaining 57, two are follow-on studies and five are performed ex vivo on isolated cells. The remaining 50 utilize *in vivo* GET for a variety of applications. Of these 50 studies, 21 are currently listed as complete. Thirty-one are in Phase 1, eight are listed as Phase 1/2, and 11 are in Phase 2. The two major applications under evaluation are cancer-related therapies (22 studies) and infectious disease vaccines (26 studies); several safety and tolerability studies were conducted in healthy individuals. The predominant route of administration is intramuscular delivery with 37 of the studies using this method. Six studies use intratumor delivery and seven use intradermal delivery. One study compares intramuscular and intradermal delivery.

2.2 Cancer

As described above, a significant application of GET is the treatment of cancer (Table 1). The major approaches evaluated include immunotherapy, cancer vaccines, and antiangiogenic therapy. The predominant approach has been therapeutic DNA vaccination with 13 of the 23 trials (one additional registered on UK clinical trial site) using this approach. Six of the studies use immunotherapy and three use an antiangiogenic approach. Fifteen of the studies use an intramuscular administration route, six use an intratumor route, and one uses an intradermal route. There are 10 Phase 1, seven Phase 1/2 and six Phase 2 studies. Currently, eight of the studies have been completed. There are seven trials testing therapies for melanoma; five trials for cervical cancer; two for head and neck squamous cell carcinoma; three for multiple types, two for prostate, and one each for Merkel cell carcinoma, chronic T-cell lymphoma, colorectal, and leukemia cancers. Results are available for nine DNA electrotransfer studies and these are summarized below.

2.2.1 Cancer Immunotherapy

The first clinical trial using GET (NCT00323206) was performed in patients with metastatic melanoma (Daud et al., 2008). The protocol was based on extensive preclinical studies that demonstrated both a therapeutic response and a resistance to new tumor growth following reinjection of cells (Heller et al., 2006; Lucas et al., 2002; Lucas & Heller, 2003). The trial

Table 1 Current clinical trials of electrotransfer for cancer

Condition	Biological intervention	Site	Status	Phase	NCT identifier
Melanoma	IL-12 pDNA	Tumor	Completed	1	NCT00323206
Melanoma	IL-12 pDNA	Tumor	Recruiting	2	NCT01502293
Melanoma	VCL-IM01 (pIL-2)	Tumor	Completed	1	NCT00223899
Melanoma	SCIB1	Muscle	Recruiting	1/2	NCT01138410
Melanoma	Xenogenic tyrosinase	Muscle	Completed	1	NCT00471133
Melanoma	AMEP	Tumor	Terminated	1	NCT01045915
Melanoma	AMEP	Muscle	Not Recruiting	1	NCT01764009
Merkel cell cancer	IL-12 pDNA	Tumor	Recruiting	2	NCT01440816
Cutaneous lymphoma	IL-12 pDNA	Tumor	Recruiting	2	NCT01579318
Prostate cancer	Tetanus toxin domain fused to PSMA	Muscle	Completed	1/2	UK112
Prostate cancer	PSA	Skin	Completed	1/2	NCT00859729
Solid tumors	HER2 and CEA fused to B subunit of *Escherichia coli*	Muscle	Completed	1	NCT00647114
Disseminated metastases	AMEP	Muscle	Terminated	1	NCT01664273
Solid tumors	V934/935	Muscle	Completed	1	NCT00753415
Cervical neoplasia	GX-188E	Muscle	Recruiting	1	NCT01634503
Cervical neoplasia	VGX-3100	Muscle	Recruiting	2	NCT01304524
Cervical neoplasia	GX-188E	Muscle	Not yet recruiting	2	NCT02139267
Cervical	VGX-3100 and INO-9012	Muscle	Recruiting	1/2	NCT02172911
Cervical	VGX-3100	Muscle	Completed	1	NCT01188850
Colorectal cancer	tetwtCEA DNA	Skin	Active	1/2	NCT01064375
Leukemia	p.DOM-WT1-37 p.DOM-WT1-126	Muscle	Unknown	2	NCT01334060
HPV-related head and neck cancer	pNGVL-4a-CRT/E7	Muscle	Recruiting	1	NCT01493154
HPV-related head and neck cancer	VGX-3100 and INO-9012	Muscle	Recruiting	1/2	NCT02163057

consisted of dose escalation of the plasmid maintaining the same GET parameters. The major objective of this Phase I trial was safety and tolerability, following GET of a plasmid encoding human interleukin-12 (IL-12). Patients had metastatic melanoma with accessible cutaneous disease that was surgically unresectable. The patients were enrolled with multiple cutaneous or subcutaneous lesions and the treatment was administered directly into two to four of these sites. Twenty-four patients were treated in seven cohorts (plasmid concentrations ranging from 0.1 to 1.6 mg/ml). All patients received three treatments on days 1, 5, and 8, which included intratumor injection of plasmid pUMVC3-hIL-12-NGVL3 delivered using GET. Patients were offered an intravenous analgesic (morphine sulfate, 1 mg) and/or anxiolytic (lorazepam, 1 mg) medications approximately 15–30 min before treatment. In addition, lidocaine was applied topically to, or injected around, each tumor site. Six 100 μs pulses at an amplitude per distance ratio of 1300 V/cm were administered with a 6-needle electrode array using a Medpulser DNA EPT System Generator (OncoSec Medical Inc., San Diego, CA). Treatment was well tolerated with only Grade 1 and Grade 2 toxicity reported. No laboratory abnormality was noted. Transient pain, erythema, and bleeding at the GET sites following treatment were the only adverse events reported, but all patients stated that the therapy was tolerable. Biopsies of treated lesions were taken on days 11, 22, and 39. Posttreatment biopsies at various times after treatment (Days 11, 22, and 39) showed proportional increase in IL-12 protein with increased plasmid dose. IL-12 expression was observed at all GET sites even at the lowest dose tested (0.1 mg/ml).

Although the study was designed as a safety trial, the secondary objective was to evaluate the response. Greater than 70% of the treated lesions regressed. In addition, of the 24 patients receiving therapy, 19 had additional sites of disease outside the treated lesions. Of these 19 patients, >10% showed complete regression of all metastases, both treated and untreated, and 42% showed disease stabilization or a partial response. Histological evaluation of the lesions revealed marked tumor necrosis and lymphocytic infiltrate.

Two of the 19 patients with untreated lesions had a complete response in distant nontreated metastases. Although they each had a large number of melanoma lesions, all lesions, the four treated and all untreated, shrank and faded away over a 6–18 month period. At approximately 18 months, several lesions were biopsied and were observed to be free of tumor cells, documenting that each had a complete response. A third patient, had complete regression of all lesions (treated and untreated) and on a follow-up CT scan, had no evidence

of disease five months post GET, after having received four cycles of dacarbazine. No new lesions at any site appeared in the complete responders or those with a stable disease. The results from this study show human IL-12 plasmid delivery to melanoma patients is safe effective, reproducible, and capable of inducing an effective antitumor immune response.

Three Phase 2 trials have been initiated based on the results from the Phase 1 study described above. These trials use the same GET intratumor protocol to deliver IL-12 plasmid to patients with melanoma (NCT01502293), Merkel cell carcinoma (NCT01440816), and chronic T-cell lymphoma (NCT01579318). In the Phase 1 trial, only one treatment cycle was administered. In the Phase 2 studies, if disease is present at six months without systemic progression, then patients can be retreated every three months. Interim results are only available for the melanoma study. No treatment-related serious adverse events or deaths have been reported. In the melanoma study, 30 patients were enrolled and received at least one cycle of treatment. As of May, 2014, 28 patients were evaluable for response at the 6 month primary time point. Evaluation of the 28 patients revealed nine patients (32%) with an objective response based on modified RECIST criteria and three (11%) with a complete response. Responses of individual lesions were also assessed. A total of 85 lesions were evaluated and stable disease was observed in 26 lesions (31%); partial response in seven lesions (8%) and complete response in 38 lesions (45%).

An important aspect of this therapeutic approach is the response of untreated lesions. There were 22 patients that presented with baseline lesions that were left untreated. Following treatment, regression of at least one untreated lesion (>30% decrease in size) was observed in 13 (59%) of these patients (http://ir.oncosec.com/company-news/).

GET has also been used to deliver a plasmid encoding interleukin-2 (IL-2) intratumorally. This was also a Phase I dose escalation clinical trial (NCT00223899) that has been completed. The treatment was administered to patients with recurring metastatic melanoma. The treatment protocol consisted of two cycles, which included four weekly treatments of intratumor plasmid injections and GET, followed by a four-week observation period. GET was administered with six 100 μs pulses and amplitude per distance ratio of 1300 V/cm (instrument). No Grade 3 or 4 adverse events were observed. Injection-site pain was the most commonly reported adverse event. The overall response data has not yet been published.

A Phase 1/2 study utilizing intramuscular GET to deliver an immunotherapeutic agent in the form of a plasmid encoding SCIB1 (NCT01138410) was conducted. This plasmid encodes a human antibody molecule

engineered to express Tyrosinase-Related Protein 2 (TRP2, a melanoma antigen) plus two helper T-cell epitopes. The construct was designed to stimulate a T-cell response against melanoma. The study was designed to enroll up to 22 HLA-type A2 patients with Stage III or Stage IV melanoma with a life expectancy of at least three months. The objective was to assess the safety and tolerability of SCIB1 as well as the safety and performance of the injection device. A secondary objective was to determine the immunological effects of SCIB1 delivery. Patients received five intramuscular doses of the SCIB1 plasmid using a device that simultaneously administers the GET protocol. The details of the protocol, including the electrode, pulse protocol, and instrument, were not available. The first part of the trial was a dose escalation (0.4, 2, 4, and 8 mg) study. Second, patients with resected disease and those who are free at the time of treatment receive the highest dose.

Interim results have been released. No dose-limiting or grade 4/5 toxicities were observed and the administration of SCIB1 was well tolerated. A clinical response was seen in 5 of 11 patients receiving 2, 4, or 8 mg doses in Part 1. The five patients receiving the high dose (8 mg) in part 1 remain alive, while survival of patients receiving the 2 mg or 4 mg doses had 1-year and 2-year were 100% and 67%, respectively. The survival rate for patients in Part 2 was 100% 16–24 months after treatment. Disease progression was observed in three patients. Cell-mediated melanoma-specific immune responses as measured by IFNγ ELISPOT assays were observed in 24 of 28 (86%) evaluable patients. Physicians have the option to continue administering SCIB1 and eight patients are currently on long-term treatment with SCIB1 (http://www.scancell.co.uk/Apps/Content/News/?id=326).

2.2.2 Cancer Vaccines

A Phase 1 study designed to evaluate the safety and immunogenicity of a DNA vaccine in melanoma patients at risk for disease progression or recurrence has been completed (NCT00471133). These patients received intramuscular GET of a plasmid encoding tyrosinase. Patients received up to five doses of the vaccine at three-week intervals. There were three dose cohorts: 0.2, 0.5, and 1.5 mg. Three patients were enrolled in the lower dose cohorts and 15 at the high dose. Minimal toxicity was observed. A grade 1 injection-site reaction was noted in 46% of the patients. In the high-dose group, 6 out of 15 (40%) developed tyrosinase-reactive CD8 cells by flow cytometry. This response was not observed at the lower doses. Three patients were found to have an increase in tyrosinase-reactive CD8 + IFNγ + T-cells by IFNγ ELISPOT assays (Yuan et al., 2013).

A Phase 1 two-part study delivering a plasmid encoding HER2 and a plasmid encoding CEA fused to the B subunit of *Escherichia coli* heat-labile toxin used intramuscular GET in patients with solid tumors (NCT00647114). Patients enrolled in the first part received up to five intramuscular injections followed by electrotransfer every 14 days. Two dose levels (0.25 and 2.5 mg) were tested. Electrotransfer included two 60 ms pulses delivered using a Medpulser device (Inovio, San Diego, CA); the amplitude per distance ratio and details of the needle electrode were not reported. The second part combined delivery of the plasmids with GET followed by a boost with an adenovirus encoding HER2 and CEA. Two dose levels of the adenoviral vector were used, 0.5×10^9 and 0.5×10^{11} vg/injection. Patients in Part 2 received GET (five vaccinations), then received injection of the adenovirus at 4 and 6 weeks after the fifth GET vaccination. A total of 28 patients were enrolled in Part 1 of the study. Eleven patients participated in Part 2 of the study; six also participated in the Part 1 and five were new to the study. Six patients received the lower adenovirus dose and five, the higher dose. Of the 28 patients enrolled in Part 1, six were at the low dose and 22 at the high dose. Twenty-seven received all five injections; one discontinued after the fourth injection due to detection of liver metastases. Of the 11 patients enrolled in Part 2, seven received all five GET administrations and both injections of adenovirus.

Results from Part 1 show that the vaccine was safe and well tolerated. The most common adverse event was injection-site pain reported by 71% of the patients. The next most common was fatigue seen in 21% of patients. Adverse events reported in Part 2 were similar to those reported in Part 1. The injection-site reaction appeared related to viral dose as it was reported in higher frequency at the higher dose. With respect to immunogenicity, no cell-mediated response was observed in either part of the study. In this study, GET was performed using short needles and the investigators suggest that it is possible that in patients with higher body mass index the needles may not have reached the muscle. There could be other reasons that no immunogenicity was observed that could be related to the GET parameters, plasmid dose, viral dose, or other factors (Diaz et al., 2013).

A Phase 1/2 study was conducted in patients with recurrent prostate cancer (registered in the UK gene therapy database UK-112; http://www.wiley.com//legacy/wileychi/genmed/clinical/). The study was designed to determine safety and immune stimulation following intramuscular electrotransfer of a DNA vaccine encoding a tetanus toxin domain fused to prostate specific membrane antigen (PMSA). The study had two arms with

16 patients in each arm. Arm 1 received the vaccine without electrotransfer at doses of 800, 1600, and 3200 μg and arm 2 received the vaccine at doses of 400, 800, and 1600 μg followed by electrotransfer. The vaccine was administered at 0, 4, 8, 24, and 48 weeks. Electrotransfer was performed using an Elgen Twinjector device (Inovio Biomedical Inc., San Diego, CA) with pulse parameters of a train of five 20 ms pulses at 8.3 Hz delivered with a maximum current of 250 mA. Brief pain was observed at the delivery site.

Adverse events were predominately at grade 1 or 2 and were related to injection-site reactions, flu like symptoms, back pain, and nail changes. There were two serious adverse events. One patient developed a grade 2 edema and another patient was admitted for a transurethral resection of the prostate. In both cases, the incidents were resolved and both continued on the study. Antibody responses to the tetanus toxin persisted up to 18 months. PMSA doubling time increased significantly post vaccination; this is an indication of slowing of disease. Immunological assessment revealed that 29 of 30 patients evaluated had a significant CD4+ response to tetanus toxin domain. IFNγ producing T-cells specific for PMSA were detected in 55% of the patients. Circulating CD8 cells specific for PMSA were only found in 20% of patients, which was not unexpected due to lack of persistence in the blood of these cells. While the differences between the two arms were not significant, there was a trend toward higher responses of both CD4 and CD8 T-cells in the GET treatment arm (Chudley et al., 2012; Low et al., 2009). Data on anti-PSMA antibodies and possible clinical efficacy have not yet been published.

A second prostate cancer study was conducted to deliver a plasmid encoding prostate specific antigen (PSA) using intradermal GET (NCT00859729). This Phase 1/2 study was designed to determine the feasibility and safety of a DNA vaccine administered intradermally followed by electrotransfer in patients with relapse of prostate cancer. The Rhesus Macaque PSA encoded by the plasmid has 89% homology to human PSA. Electrotransfer was conducted using the DERMA VAX™ (Cellectis Therapeutics, Paris, France) delivery system. The electrical parameters were two 0.05 ms pulses at an amplitude of 1125 V/cm followed by eight 10 ms pulses at an amplitude of 275 V/cm. Pulses were administered through two parallel rows of 2 mm needles (6 needles/row) separated by 4 mm spacing. There were five cohorts who received five increasing doses of plasmid DNA ranging from 50 to 1600 μg at four-week intervals. Patients received standard androgen deprivation therapy (ADT) prior to vaccinations. No systemic toxicity was observed. Based on a 10-point Visual Analog

Scale (VAS), discomfort during pulsing was graded at a mean/median of six. There were grade 1 skin reactions at the vaccination site with some patients exhibiting erythema but no induration or ulceration.

All patients exhibited increase in PSA levels during the 5-month period of vaccination. ELISPOTS were used to assess vaccine efficacy. In the first three cohorts, no response against the vaccine-specific antigen was seen although some patients in cohorts 2 and 3 had increases in preexisting PSA responses. Responses may have been masked due to increased T-cell activity induced by ADT. Vaccine-specific responses were seen in two patients each from cohorts 4 and 5 (Eriksson, Totterman, Maltais, Pisa, & Yachnin, 2013).

2.2.3 Antiangiogenesis

Three Phase 1 clinical trials (NCT01664273; NCT01764009; NCT01045915) have been designed to treat cancer using an antiangiogenesis approach. The populations were either patients with melanoma or with any metastatic disease. All three trials were designed to deliver a plasmid encoding antiangiogenic metargidin peptide (AMEP) using either intratumor or intramuscular delivery. This plasmid is designed to downregulate two integrins, $\alpha 5\beta 1$ and $\alpha v\beta 3$. Results are available for one of the studies (NCT01045915), which utilized intratumor delivery of plasmid AMEP to patients with melanoma. The primary objective of the trial was to evaluate the local and general safety of the procedure using increasing doses of DNA. The secondary objective was to identify doses that could be effective on cutaneous lesions. Each patient had two lesions identified; one for treatment and one to be observed as an untreated control. The study was initially proposed to test four doses of plasmid at 1, 2, 4, and 6 mg divided into two separate administrations given one week apart with three patients at each dose level. Patients received local lidocaine injections. Electrotransfer was administered immediately after plasmid injection. The pulse protocol consisted of one 100 μs pulse at an amplitude per distance ratio of 1250 V/cm followed 1 s later by one 400 ms pulse at an amplitude per distance ratio of 140 V/cm. These pulses were administered through a linear-needle electrode with a 4 mm gap between the two rows of four needles using a Cliniporator pulse generator (IGEA, Carpi, Italy).

Due to slow recruitment, the study was terminated after the fifth patient was enrolled. The procedure was found to be safe and well tolerated with no serious adverse events reported. Three patients experienced erythema around the injection site, one patient had a transient fever, and two patients had a transient increase in C-reactive protein. The electrotransfer procedure was well tolerated with a mean VAS score of 0.6 (0–10 scale). The five

treated lesions were reported to be stable with <20% increase in size, while four of the five control lesions increased over 20% with no response seen in any distant lesions (Spanggaard et al., 2013).

2.3 DNA Vaccines for Infectious Agents

DNA delivery is an appealing approach to infectious disease vaccines and this has been a major area of growth for GET. Conventional infectious disease vaccines involve pathogen cultivation, a risk itself, and vaccine delivery may create the potential for infection in immunosuppressed and immunocompromised individuals. DNA vaccines can be produced quickly to manage outbreaks or genetically changing organisms. However, plasmid DNA injection alone is only weakly immunogenic, probably due to insufficient delivery and expression (Kutzler & Weiner, 2008). As discussed above, electrotransfer is a reliable method of increasing transgene expression levels.

Many infectious disease vaccines have been tested in preclinical models. Increased levels of antigen expression and specific immunity are induced after electrotransfer of virtually all the plasmids encoding viral, bacterial, or parasitic antigens tested. The vast majority of clinical trials evaluating DNA vaccines delivered by electrotransfer are against viral agents; one clinical trial is against a parasitic agent.

There are currently 26 clinical trials listed on ClinicalTrials.gov using this approach (Table 2). In 23 of the trials, injection of plasmid DNA encoding specific antigens into the deltoid muscle was followed by pulse delivery using needle-type electrodes. In three trials, DNA encoding specific antigens was injected intradermally followed by pulse delivery using very short needles. One study is comparing these two routes of administration. Currently there are 18 Phase 1, three Phase 1/2, and five Phase 2 studies. Ten of the trials have been completed. With respect to targets, there are 11 studies for human immunodeficiency virus, five for influenza, three for Hepatitis C, one for human papillomavirus, two for Hepatitis B, two for Haantaan and Puumula viruses, one for Venezuelan Equine Encephalitis virus, and one for the malaria-inducing protozoan parasite *Plasmodium falciparum*. Results are available for seven of the studies and those are summarized below.

2.3.1 Human Immunodeficiency Virus

Two groups have pursued electrotransfer of human immunodeficiency virus (HIV) vaccines in healthy volunteers. The first published trial expanded on a previous trial in which a 1:1 mixture of plasmids encoding HIV Clade C/B′ gag and env and encoding HIV Clade C/B′ pol and nef-tat fusion antigens

Table 2 Current clinical trials using electrotransfer for DNA vaccines for infectious agents

Condition	Biological intervention	Site	Status	Phase	NCT identifier
HIV	HIV-MAG vaccine VSV HIV vaccine IL-12 pDNA	Muscle	Active	1	NCT01578889
HIV	PENNVAX-B IL-12 pDNA	Muscle	Completed	1	NCT00991354
HIV	HIV-MAG vaccine GENEVAX® IL-12	Muscle	Completed	1	NCT01266616
HIV	HIV-MAG vaccine GENEVAX® IL-12 Ad35-GRIN/ENV	Muscle	Completed	1	NCT01496989
HIV	HIV-1 multi-antigen vaccine IL-12 pDNA	Muscle	Recruiting	1	NCT01859325
HIV	HIVIS DNA vaccine	Skin	Recruiting	2	NCT01697007
HIV	PENNVAX-B	Muscle	Completed	1	NCT01082692
HIV	ADVAX	Muscle	Completed	1	NCT00545987
HIV	DNA Vaccine	Muscle	Active, not recruiting	2	NCT01705223
HIV	GTU-multiHIV B clade	Muscle	Recruiting	1	NCT02075983
HIV	PENNVAX-G MVA-CMDR	Muscle	Active, not recruiting	1	NCT01260727
Influenza	VGX-3400X	Muscle	Completed	1	NCT01142362
Influenza	INO-3605; INO-3609; INO-3401; INO-3605 & INO-3609; INO-3510	Skin	Active	1	NCT01405885
Influenza	VGX-3400	Muscle	Enrolling	1	NCT01184976
Influenza	FVH1	Skin	Active	1	NCT01587131

(*Continued*)

Table 2 Current clinical trials using electrotransfer for DNA vaccines for infectious agents—cont'd

Condition	Biological intervention	Site	Status	Phase	NCT identifier
Influenza	INO-3401	Skin	Completed	1	NCT01403155
Chronic hepatitis C	CHRONVAC-C	Muscle	Active, not recruiting	1/2	NCT00563173
Chronic hepatitis C	CHRONVAC-C	Muscle	Recruiting	2	NCT01335711
Chronic hepatitis C	VGX-6150	Muscle	Recruiting	1	NCT02027116
Chronic hepatitis B	HB-110	Muscle	Completed	1	NCT01641536
Chronic hepatitis B	HBV DNA vaccine	Muscle	Unknown	2	NCT01487876
HPV	VGX-3100	Muscle	Completed	1	NCT00685412
Hantaan and Puumala viruses	HTNV/PUUV	Muscle	Active not recruiting	2	NCT02116205
Hantaan and Puumala viruses	pWRG/HTN-M(x); pWRG/PUU-M(s2)	Muscle	Completed	1	NCT01502345
Malaria	EP-1300	Muscle	Completed	1	NCT01169077
Venezuelan equine encephalitis	VEE DNA	Skin and muscle	Recruiting	1	NCT01984983

were injected intramuscularly (NCT00249106) (Vasan et al., 2010). Without electrotransfer, low-level transient cellular immune responses were observed. In the electrotransfer trial (NCT00545987) (Kopycinski et al., 2012; Vasan et al., 2011), cohorts of patients received 0.2, 1.0, or 4.0 mg of plasmid mixture or placebo; injection depth was adjusted based on skin-fold thickness. Immediately after injection, 24 patients received three 40 ms pulses at an amplitude to distance ratio of 250 V/cm over a 400 ms interval using a TriGrid Delivery System (Ichor Medical Systems, San Diego, CA, USA), with a 6 mm needle array in a diamond formation.

There were no study-related serious adverse events. A significant increase in patients reporting mild to moderate local pain or tenderness at 30–45 min was observed with the addition of electrotransfer over injection alone, although after four days, no significant difference was reported. When asked, 97% of patients agreed that electrotransfer was acceptable for vaccine delivery for life-threatening infections; for nonlife-threatening illnesses, this acceptance dropped to 91%. Immunogenicity analysis showed that electrotransfer produced a 70-fold increase over injection alone in specific cell-mediated immunity to the env antigen as determined by IFNγ ELISPOT assays. Increases of 22, 13, and 19 fold were reported for the pol, gag, and nef-tat antigens, respectively. No specific humoral immunity was detected.

A second HIV vaccine clinical trial (NCT00991354) in healthy adults combined a plasmid encoding HIV Clade B env, gag, and pol proteins with a plasmid encoding the human IL-12 subunits to enhance cellular immune responses (Kalams et al., 2013). This trial also expanded on a previous trial (NCT00528489) testing intramuscular plasmid injection alone. Ten subjects received 3 mg HIV plasmid, 30 subjects received 1 mg of combined plasmids, and eight subjects received placebo in a series of intramuscular injections on days 0, 28, and 84 of the study. After each intramuscular plasmid injection, three 52 ms pulses at a constant current of 0.5 A at a frequency of 1 Hz were applied (CELLECTRA Device, Inovio, Blue Bell, PA, USA).

There were no study-related serious adverse events. All participants experienced some pain with pulse delivery; the median on the VAS was 5.0–5.4 of 10. After the third vaccination, 66% of patients would willingly undergo electroporation as a prevention component for a serious disease. This percentage dropped to 27% of patients willing to undergo electroporation to increase the effectiveness of an existing vaccine. Almost 90% of individuals developed CD4+ or CD8+ T-cell responses after electrotransfer. The specific humoral immunity induced was minimal. Interestingly, the T-cell response rate depended on age and body mass index (BMI),

presumably because at larger BMIs, the deltoid muscle was beyond the depth of the 18 mm electrodes.

2.3.2 Hepatitis Viruses

Hepatitis B virus (HBV) causes a wide range of liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. In a Phase II clinical study (NCT01189656), electrotransfer of a DNA vaccine was evaluated in patients with HBeAg-positive chronic hepatitis B infections. The vaccine consisted of two plasmids encoding the HBV middle envelope protein and an adjuvant plasmid encoding a human IL-2/IFNγ fusion protein. This DNA vaccine was tested as a monotherapy in six patients whose serum alanine aminotransferase levels were not indicative of antiviral treatment. The vaccine was also tested in combination with lamivudine therapy (100 mg daily, GlaxoSmithKline Pharmaceuticals, Suzhou, Jiangsu Province, China) in 22 patients. Eleven control patients received lamivudine plus placebo (Yang et al., 2012). Each dose of 4 mg plasmid (2 mg DNA vaccine plus 2 mg adjuvant plasmid) was injected into four sites in each deltoid muscle; injections were performed on weeks 0, 4, 12, and 24. Pulses were applied with a custom square-wave pulse generator using two silver acupuncture needles 1 cm apart. The pulse parameters were unstated.

There were no study-related serious adverse events. Only mild adverse effects were noted, including tolerable pain, self-limited redness, and swelling around the injection site. No serum cytokines were detected during the entire course of the trial. In patients receiving the DNA vaccine as a monotherapy, a significant elevation in specific T-cell response was indicated by IFNγ ELISPOT assays. In patients receiving combination therapy, a suppression of serum HBV DNA copy number was also detected indicating the suppression of viral replication.

Chronic hepatitis C virus (HCV) infection also causes severe liver disease and hepatocellular carcinoma. Clearance of HCV requires specific T-cell function. In this clinical trial (NCT00563173), the vaccine plasmid encoded the hepatitis C genotype 1a nonstructural (NS) 3/4A gene, one of the most conserved HCV core genes (Weiland et al., 2013). Twelve patients with verified chronic HCV genotype 1 infections of a duration >12 months received four monthly vaccinations of 167 μg (three patients), 500 μg (three patients), or 1500 μg (six patients) plasmid DNA in alternating deltoid muscles. Two 60 ms pulses at an amplitude per distance ratio of 106 V/cm were administered using a 4-electrode array (Medpulser DDS, Inovio, Blue Bell, PA, USA).

No study-related serious adverse events were noted. Subjects noted transient pain with a mean of approximately 5 of 10 on a relative scale accompanied by two small muscle twitches. This vaccine transiently increased relative specific antibody levels, specific T-cell response as indicated by IFNγ ELISPOT assays. Transient changes in viral load were observed in eight patients; three had increased levels, while five had reduced levels in serum HCV RNA. After the immunological studies, 8 of the 12 patients also received standard of care treatment, pegylated interferon, and ribavirin. The authors suggest that this vaccination may hold promise as a combination therapy.

2.3.3 Human Papillomavirus 16/18

Current effective human papillomavirus (HPV) vaccines target capsid antigens and prevent infection with subtypes 16 and 18 subtypes responsible for the majority of HPV-caused cancers. The vaccine in this study was designed to prevent disease progression in patients previously treated for high-grade cervical dysplasia. In this vaccine trial (NCT00685412), the plasmids encoded the consensus E6 and E7 genes of HPV subtypes 16 and 18. These antigens are expressed in infected cells. Patient cohorts received a three dose series of intramuscular injections containing 0.6, 2, or 6 mg of plasmid DNA at months 0, 1, and 3. Pulses were applied using a constant current device (CELLECTRA, Inovio, Blue Bell, PA, USA) (Bagarazzi et al., 2012). The pulse protocol was unstated but may be similar to another published trial (Kalams et al., 2013). There were no study-related serious adverse events. Most participants experienced some pain with pulse delivery; the mean on the VAS was 6.2 of 10, which dissipated to 1.4 of 10 within 10 min. Antibody titers to HPV16 increased in 67% of patients; titers to HPV18 increased in 39% of patients and persisted at least 6 months after the treatment regimen. By standard IFNγ ELISPOT assays, specific CD8+ T-cells were detected in four of six subjects receiving the 0.3 mg plasmid dose, in five of six subjects receiving the 1 mg plasmid dose, and in five of six subjects receiving the 3 mg plasmid dose. Full cytotoxic functionality was induced in all cohorts.

2.3.4 Hantaviruses

Hantavirus infections causing hemorrhagic fever with renal syndrome are endemic in Europe and Asia. A Phase I clinical trial in 31 healthy adult volunteers (NCT02116205) focused on vaccines encoding the Gn and Gc envelope glycoprotein genes of two hantaviruses, Hantaan virus and Puumala virus (Hooper et al., 2014). After each of three intramuscular injections totaling 2 mg DNA of Hantaan virus vaccine, Puumala virus vaccine, or the

combination, electric pulses were applied (TDS-IM Device, Ichor Medical Systems, San Diego, CA, USA). Neither the electrode nor the pulse protocol employed was stated. There were no study-related serious adverse events. Only two subjects reported muscle contractions with pulse application. Twenty-eight subjects reported local pain at the injection site; seven patients also developed bruising. Five of nine and seven of nine individuals developed neutralizing antibodies when the Hantaan virus and Puumala virus plasmids were delivered as single vaccines. When the combination was tested, seven of nine developed neutralizing antibodies to Puumala virus, while a reduced number (three of nine) developed antibodies to Hantaan virus.

Other trials may be in progress but are not yet published or recorded in this Clinical Trials registry. In several trials, the electrodes and electroporation parameters used for delivery were not detailed. Changing pulse parameters (pulse number, intensity, length, and frequency) causes variations in the levels and duration of transgene expression. The pulse parameters are a critical part of the delivery criteria and should be clearly delineated when the results of each trial are published.

In each of these trials, patients universally complained of local, transient pain due to pulse application. However, in general, the pulses were well tolerated.

These studies polled patients after a complete treatment regimen with intramuscular electrotransfer using different pulse protocols. The different protocols may explain the patients' varying responses from the patients.

2.4 Safety and Tolerability

Several nontherapeutic clinical trials gauging the tolerability of the pulse types used for plasmid delivery with electroporation have been performed (Table 3). After an early study demonstrating that application of the exponential decay pulse form without drug or anesthetic was tolerable

Table 3 Current clinical trials for safety and tolerability

Tissue	Biological intervention	Status	Phase	NCT identifier
Skin	Safety and tolerability of Derma Vax No agent administered	Completed	1	NCT01324843
Muscle	Safety and tolerability of delivery of V930	Completed	1	NCT00721461
Skin	Safety and tolerability of Cliniporator	Completed	1	Not registered
Skin and muscle	Safety and tolerability of Cellectra device	Completed	1	Not registered

(Zhang & Rabussay, 2002), subsequent studies employed square-wave pulses. Two primary tissues were targeted, the skin and the deltoid muscle.

In an early skin-pulse delivery study, eight 100 μs 600 V/cm pulses at two pulse frequencies (1 and 5 Hz) were delivered with a caliper electrode to the forearm of 40 healthy volunteers using a Cliniporator (IGEA, Carpi, Italy). Three additional volunteers opted not to complete the experiment. There were no study-related serious adverse events. A wide variation in pain intensity with a range of 6–94 mm on a 100 mm VAS scale was reported. At 1 Hz, each individual pulse was associated with an instantaneous involuntary muscle contraction, while at 5 kHz only one muscle contraction was reported. While pain intensity did not vary with pulse frequency, subjects preferred a higher frequency and shorter delivery time (Zupanic, Ribaric, & Miklavcic, 2007).

In a more recent study, 10 healthy adults received three randomized pulse protocols delivered to the skin of the deltoid region (El-Kamary et al., 2012). The three protocols included short, high-intensity pulses (six 100 μs pulses at an amplitude per distance ratio of 1700 V/cm at a 10 Hz frequency), longer, low-intensity pulses (six 10 ms pulses at 200 V/cm at a 10 Hz frequency), and a combination of pulse types (one 50 ms pulse at 1125 V/cm at a 5 Hz frequency plus one 50 ms pulse at 1125 V/cm at a 16.67 Hz frequency plus eight 10 ms pulses at 200 V/cm and a 16.67 Hz frequency) delivered using an array of eight rows of 10 needles each at an interneedle distance of 600 μm (EasyVax, Cellectis Therapeutics, Paris, France). Patients were followed up to 48 h. There were no study-related serious adverse events. No significant difference was observed in tolerability between the three pulse protocols, never exceeding a 12 of 100 VAS score. Pain and tingling resolved within 5 min.

One study focused on delivery to the deltoid muscle. After injecting PBS into the deltoid muscle, 24 subjects received two 60 ms pulses at an amplitude of approximately 200 V/cm (MedPulser DDS, Inovio Biomedical Inc., San Diego, CA) with a rectangular array of 4 needles (Wallace et al., 2009). Pain severity was evaluated at the time of pulse delivery and for 14 days after treatment. There were no study-related serious adverse events. The peak discomfort was reported within 5 min of the delivery, peaking at 2.3 on the McGill Pain Questionaire scale of 1–5, or 4.75 on the Brief Pain Inventory scale of 1–10. In addition, erythema at the electroporation site was reported in 58.3% of subjects.

The tolerability of both skin- and muscle-pulse delivery in healthy volunteers was tested using a constant current instrument (CELLECTRA

Device, Inovio, Blue Bell, PA, USA) (Diehl et al., 2013). Sterile saline was injected into the deltoid muscle or intradermally in the back of the arm. Intramuscular delivery required a BMI less that 30 kg/m^2. The electrodes and pulse protocols varied based on the tissue. For intramuscular applications, three 52 ms pulses at a frequency of 1 Hz and a current of 0.5 A were delivered with a 5-needle array. For intradermal delivery, four 52 ms pulses at a frequency of 5 Hz and a current of 0.2 A were delivered with 3-needle array. There were no study-related serious adverse events. Muscle-pulse delivery (mean of 7 out of 10 VAS score) was scored as more painful than skin delivery (mean of 3 out of 10 VAS score), although in each case, the pain was short-lived.

Short-term pain or discomfort is associated with the application of electric pulses. This transient pain varies depending on the electrical parameters, the electrode used, and site of pulse delivery. It may be diminished or ultimately eliminated by reducing the distance between electrodes, thereby decreasing the applied voltage, by varying the pulse frequency, or by other means. In both healthy volunteers and clinical studies of vaccines, this pain is deemed acceptable for treatment of diseases for which alternative approaches are not effective. In cancer studies, this transient pain is a welcome alternative to the long-term pain associated with surgical intervention.

3. SUMMARY AND CONCLUSIONS

The previous two decades have seen tremendous growth in the use of *in vivo* electrotransfer. Publications and citations have continually increased during this time (Figure 1). Originally, in the 1980s, electrotransfer was used as an effective laboratory tool to deliver DNA to cells *in vitro* (Neumann, Schaefer-Ridder, Wang, & Hofschneider, 1982). A major shift in the field occurred in the late-1980s when it was shown that electrotransfer could be safely applied *in vivo* in delivering molecules to cells within tissues (Belehradek, Orlowski, Poddevin, Paoletti, & Mir, 1991; Mir, Orlowski, Belehradek, & Paoletti, 1991; Okino & Mohri, 1987). The first use of this approach was to deliver chemotherapeutics (electrochemotherapy), which rapidly progressed from preclinical animal studies to human clinical studies (Belehradek et al., 1993; Heller et al., 1998; Mir et al., 1998). Electrochemotherapy has been approved for use in Europe and is currently being used in over 300 hospitals (Cadossi et al., 2014). The next major shift came in the mid-1990s when it was demonstrated that electrotransfer could be

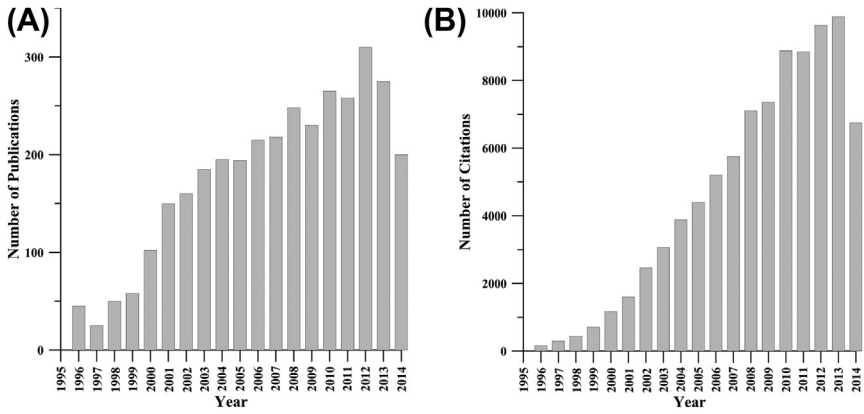

Figure 1 ***Publication and Citation Trends for* in vivo *Electrotransfer*.** Results of a search conducted on Web of Science (Thomson Reuters, http://wokinfo.com/) using the terms "*in vivo* electrotransfer," "*in vivo* gene electrotransfer," "*in vivo* electroporation," "*in vivo* electroinjection," and "*in vivo* electrogenetransfer." (A) Publications per year from 1996 to June 2014. (B) Citations per year for years 1996 to June 2014.

used safely to deliver plasmid DNA *in vivo* to cells within tissues (Heller et al., 1996; Nishi et al., 1996). Within 10 years, the first clinical trial utilizing this approach to deliver plasmid DNA was initiated (Daud et al., 2008).

Electrotransfer is a physical delivery approach, and as such, it is feasible to use it to deliver molecules to any accessible cell or tissue, provided the correct delivery parameters are utilized. Due to the diverse tissue targets, the use of GET has grown both in the number of investigators using the approach and in the number of applications being tested. This steady growth is related to the versatility inherent in this delivery system as it can be used to deliver plasmid DNA for multiple applications, and importantly, expression can be controlled through variation of electrotransfer parameters. The number of publications continues to increase and document the effectiveness of GET (Andre & Mir, 2010; Aurisicchio, Mancini, & Ciliberto, 2013; Bodles-Brakhop et al., 2009; Fioretti, Iurescia, Fazio, & Rinaldi, 2013; Gothelf & Gehl, 2010; Heller & Heller, 2006; Sardesai & Weiner, 2011). The diversity is apparent as both localized and systemic effects can be induced, so the approach is applicable to many diseases.

As mentioned above, an important consideration for GET is the pulse parameters used. The critical parameters include tissue target, electrode configuration, applied voltage, pulse width, and pulse number. Selection of these variables can influence the expression profile of the delivered transgene. Once an investigator has determined the appropriate expression

required for a particular application, the parameters can be manipulated to achieve a particular expression pattern, for example, high- or low-expression level, short or extended duration, and the requirement for local or systemic expression. Understanding the relationship between pulse parameters and expression is critical to the success of therapeutic applications. This emphasizes the importance for investigators to publish the parameters to allow for direct comparisons of protocols for same or different applications. Unfortunately, this is not common practice in the literature as evidenced by several of the studies reported in this chapter that did not state all of the GET parameters.

While the first publications specifically using an *in vivo* approach in delivering DNA were published in the mid-1990s, it was not until the late 1990s that the first reports of the delivery of plasmids with therapeutic potential were reported (Kreiss, Bettan, Crouzet, & Scherman, 1999; Niu et al., 1999; Rizzuto et al., 1999). Since then, applications using GET have grown significantly. However, this area is still relatively young with respect to clinical translation. The first clinical trial was initiated in 2004 (Daud et al., 2008) and now over 50 trials use GET for direct gene delivery *in vivo* or ex vivo in several applications. It is clear from the published reports that GET can be safely administered. Three administration routes, intramuscular, intratumor, and intradermal, have been evaluated in clinical trials. The major application has been immune stimulation either in the form of vaccines or immunotherapy. Therapeutic approaches have focused on cancer and early results indicate that some applications have demonstrated level of efficacy. Immune stimulation as indicated by increased antibody levels and/or increased cell-mediated immunity markers have been demonstrated in several of the DNA vaccine studies.

As more investigators use GET, publish their findings, and indicate the pulse parameters used, investigators new to the field will be able to use this approach using established protocols in the literature. Another consequence of the increased use of GET is the increased availability of equipment and electrodes. Investigators should be cautious as even though using established protocols may accelerate research efforts, it is important to confirm that the established protocol can be performed using similar equipment and with the same parameters and tissue target and will yield the appropriate expression profile.

It is evident from the increase in clinical trials during the past several years and by the further increase in successful preclinical studies that the number of clinical studies using GET will continue to increase. Therapeutic approaches

have included DNA vaccines for prophylaxis or for therapeutic applications, immunotherapeutic agents, cell-growth inhibitors, pro-apoptotic agents, tumor antigens, and antiangiogenic agents. Many studies have evaluated GET protocols for new potential therapeutic applications to skin and muscle and also to new tissue targets including the lung, heart, eye, liver, and cochlea (Ayuni et al., 2010; Chi et al., 2005; Dean, 2013; Ferraro, Cruz, Baldwin, Coppola, & Heller, 2010; Ferraro, Cruz, Coppola, & Heller, 2009; Hargrave et al., 2013; Heller, Cruz, Heller, Gilbert, & Jaroszeski, 2010; Jaichandran et al., 2006; Lee, Chesnoy, & Huang, 2004; Lin & Dean, 2011; Marshall et al., 2010; Marti et al., 2004; Marti et al., 2008; Martin, Young, Benoit, & Dean, 2000; Pinyon et al., 2014; Sakai, Nishikawa, Thanaketpaisarn, Yamashita, & Hashida, 2005; Touchard et al., 2009, 2010, 2012; Zhou, Norton, Zhang, & Dean, 2007).

The number of completed or active trials using GET is relatively small compared to other gene transfer techniques. However, the last few years have seen multiple published reports documenting successes in these early clinical studies. The increase in the number of preclinical studies showing therapeutic success suggests that there will be a significant increase in the number of clinical trials using GET in the near term. The true indication of how successful this approach is will not be known until more studies are published or one or more of these therapeutic or prophylactic protocols obtain regulatory approval.

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INDEX

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COLOR PLATES

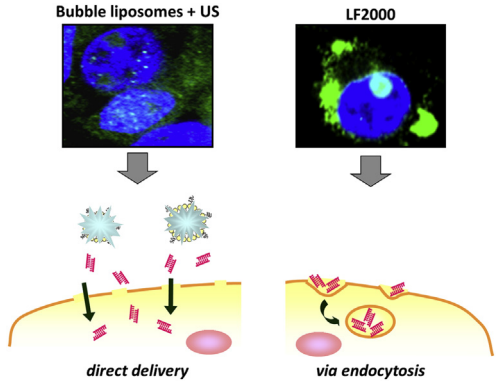

PLATE 1 ***Mechanism of action of bubble liposomes (BLs) and ultrasound (US).*** Localization of FITC-siRNA after transfection using BLs and US, or Lipofectamine2000 (LF2000), and differences in their mechanism of action. *Reprinted from Negishi et al. (2008) with permission.* (Figure 6 on page 34 of this Volume)

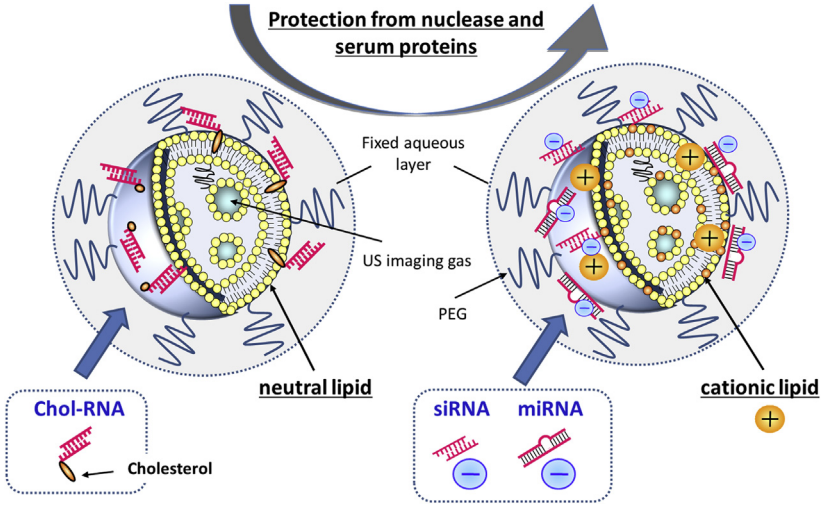

PLATE 2 ***Schematic showing a nucleic acid-loaded bubble liposomes (BL).*** (Figure 7 on page 35 of this Volume)

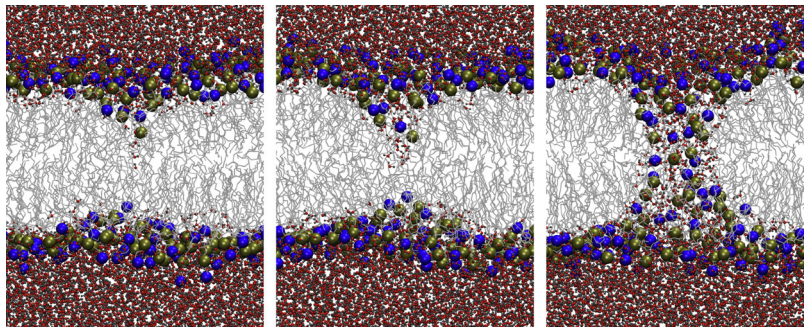

PLATE 3 ***Molecular dynamics solution of the formation of hydrophilic water channels in a membrane in response to an applied electric field.*** Snapshots of the time evolution of water–lipid–water configurations under an external electric field of 500 MV/m. Panels (left to right) are 5.8, 6.7, and 7.3 ns from the start of the simulation with both water molecules (oxygen–red, hydrogen–gray) and lipid molecules (phosphorus–yellow, nitrogen–blue, lipid tail groups–silver) displayed. *Reprinted with permission from Tokman et al. (2013).* (Figure 2 on page 52 of this Volume)

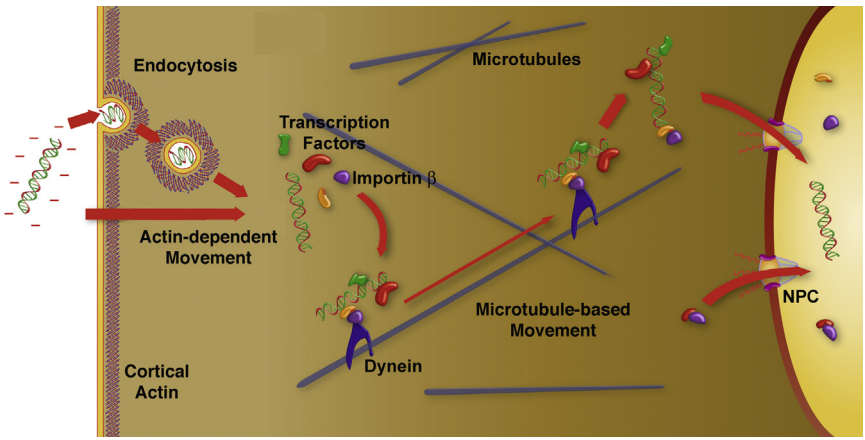

PLATE 4 ***Postelectroporation trafficking of plasmids to the nucleus during gene transfer.*** Following electropermeabilization of the membrane, plasmids may enter the cell by either endocytosis and/or direct entry into the cytosol (Rosazza et al., 2013; Rosazza et al., 2011). The initial trafficking events near the cell surface appear to involve actin and actin-based movement. Once through the cortical actin layer and free in the cytoplasm, plasmids are rapidly complexed by a number of DNA-binding proteins present in the cytoplasm which in turn bind to other proteins to form large protein–DNA complexes (Badding et al., 2013). Transcription factors bound to DNA interact with importin β and other proteins that in turn link the complex to dynein for movement along microtubules to the nucleus where it falls apart at the nuclear periphery (Badding et al., 2012). Nuclear entry is then mediated by importin β in a sequence- and importin-dependent manner. (Figure 3 on page 56 of this Volume)

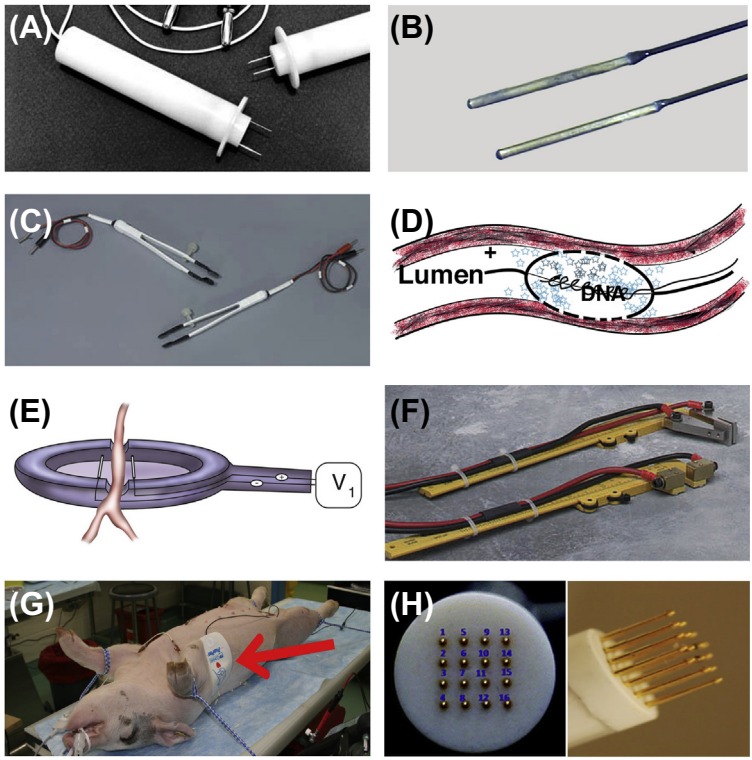

PLATE 5 ***Examples of electrodes for electroporation.*** (A) Penetrating, two-needle arrays. (B) Nonpenetrating parallel needles (Genetrode electrodes, Genetronics, San Diego, CA, USA). (C) Plate electrodes (Tweezertrodes, BTX, Hollister, MA). (D) Cartoon of a balloon catheter-based electrode for delivery of DNA and electroporation. (E) Spoon electrode for vascular electroporation. (F) Caliper-mounted plate electrodes. (G) Conformable defibrillator pads for electroporation (arrow). (H) Multielectrode array (R. Heller, personal communication). (Figure 4 on page 61 of this Volume)

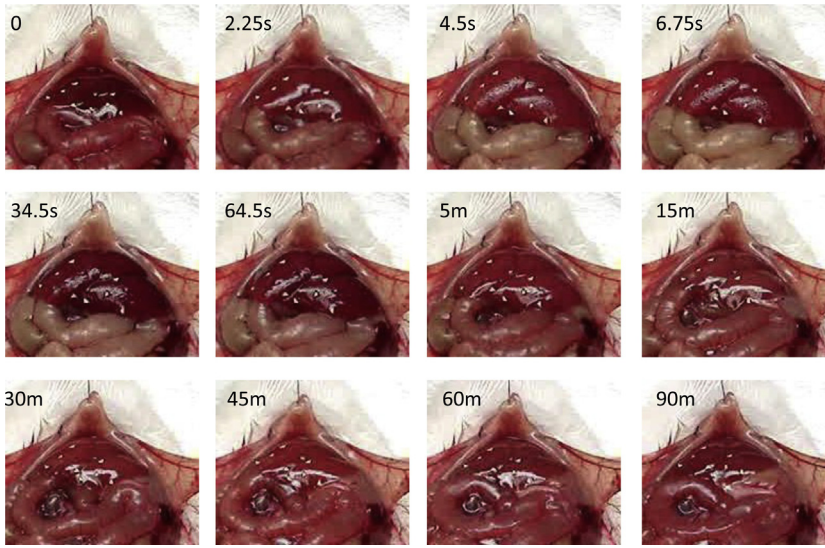

PLATE 6 ***Impact of hydrodynamic injection on liver volume.*** Changes in volume and appearance of the liver were digitally captured through an open abdomen during and after the injection from the inferior vena cava in an anesthetized mouse, and representative snapshots are presented before (0), during (2.25 s), at the end of (4.5 s), and thereafter (6.75 s–90 m) the injection. The whitish triangular patches on the liver surface are markers attached for photogrammetry of the liver volume (Suda et al., 2007). The units "s" and "m" represent seconds and minutes, respectively. (Figure 2 on page 92 of this Volume)

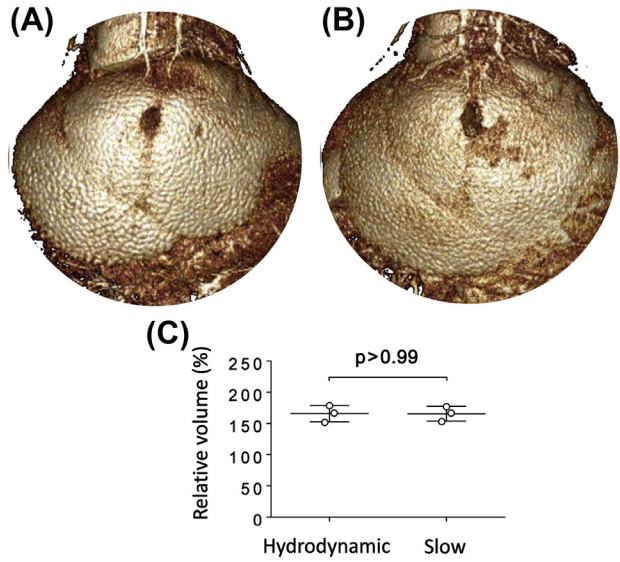

PLATE 7 ***Liver volume difference between hydrodynamic and slow injections.*** Mice receiving hydrodynamic (A, 5 s) or slow (B, 60 s) injection of contrast medium equivalent to 9% body weight were subjected to a cone beam-computed tomography scan immediately after the conclusion of the injection, and maximum intensity projection images were reconstructed based on the three-dimensional volumetric data. The entire liver volumes were significantly enlarged after injection, but there were no significant differences between hydrodynamic (165.6 $\pm$ 13.3%) and slow (165.5 $\pm$ 11.9%) injections. The liver volume is expressed as a percentage of the volume from three control mice without injection in mean $\pm$ SD (C, $p > 0.99$, Mann Whitney test). *Figure 4(C) is taken from Kanefuli et al. (2014)*. (Figure 4 on page 94 of this Volume)

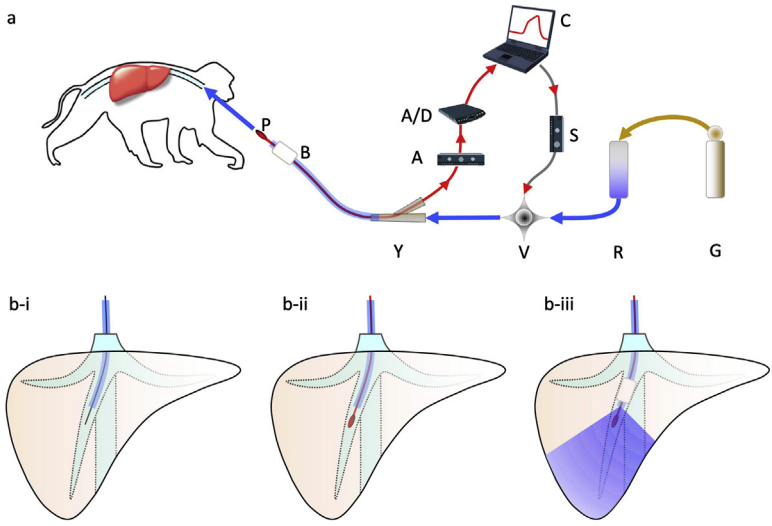

PLATE 8 ***Schematic presentation of computer-controlled, image-guided hydrodynamic delivery to the liver.*** A balloon catheter is inserted under fluoroscopic guidance, and its tip is placed at a proper point in the hepatic vein (b–i). A pressure transducer (P) is coaxially inserted through a Y-shape connector (Y) (b–ii). Inflating the balloon (B), high-pressure CO_2 (G) propels DNA solution in a reservoir (R) to splash the solution into the target area (b–iii). The signal of elevated intravascular pressure is amplified (A) and sent to the computer (C) through an analogue/digital converter (A/D), controlling the power supply (S), and manipulating the valve (V) opening and closure to match the pressure with a preloaded profile. (Figure 5 on page 101 of this Volume)

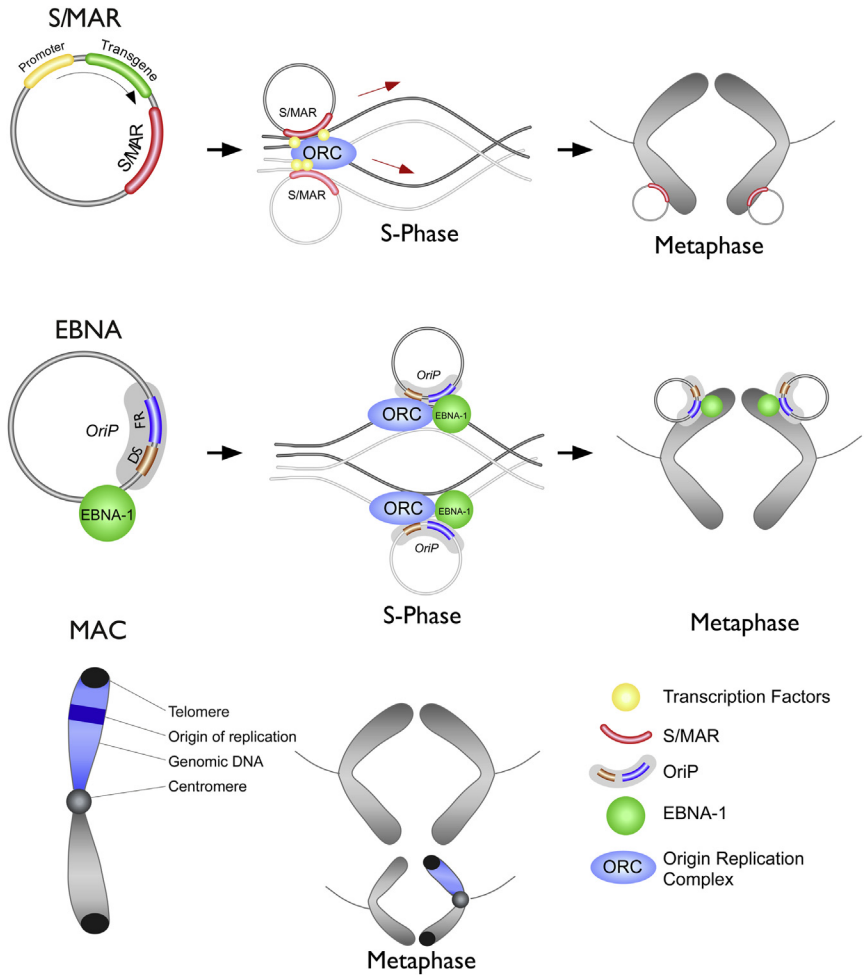

PLATE 9 ***Schematic representation of the elements and mechanisms which mediate the episomal retention of DNA vectors.*** The different mechanisms of mitotic stability of S/MAR DNA vectors, EBV vectors, and MACs in replicating cells mediated by association with the chromosomes (EBV- and S/MAR-based vectors) or interaction with spindle fibers (MACs). Compared to EBV, the S/MAR DNA vector does not require viral proteins for its replication and segregation. DS, dyad symmetry; EBNA-1, EBV nuclear antigen-1; FR, family of repeat; oriP, origin of plasmid replication. (Figure 1 on page 120 of this Volume)

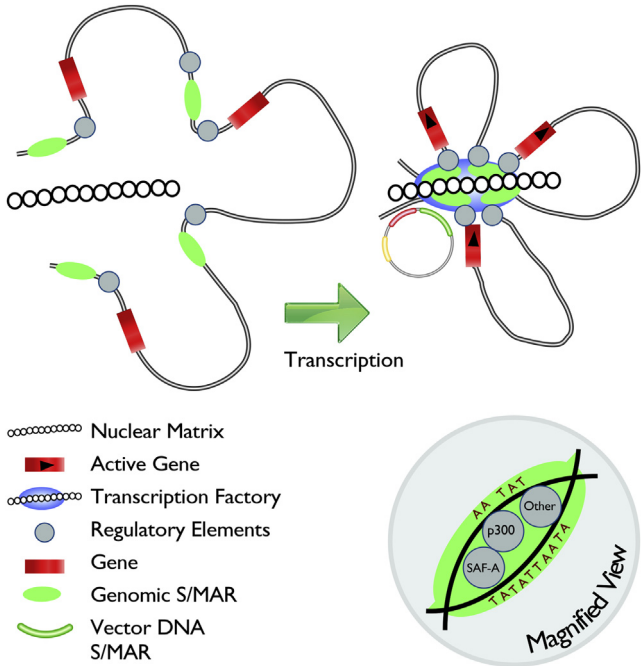

PLATE 10 ***Schematic model depicting function of S/MAR in gene regulation.*** The activation of transcription is accompanied by the anchoring of S/MAR motifs to the nuclear matrix. This results in the formation of anchored chromatin loops that are insulated from the stimulatory or repressive effects of flanking chromatin. The transcription machinery is assembled at the site of S/MAR and brings together gene-coding sequences and regulatory elements, thus enabling coordinated regulation of specific genes. At the end of the S-phase, the replication machinery is dismantled. Magnified view illustrates the interaction of the AT-rich S/MAR motifs to components of the nuclear scaffold (Fiorini et al., 2006). (Figure 2 on page 126 of this Volume)

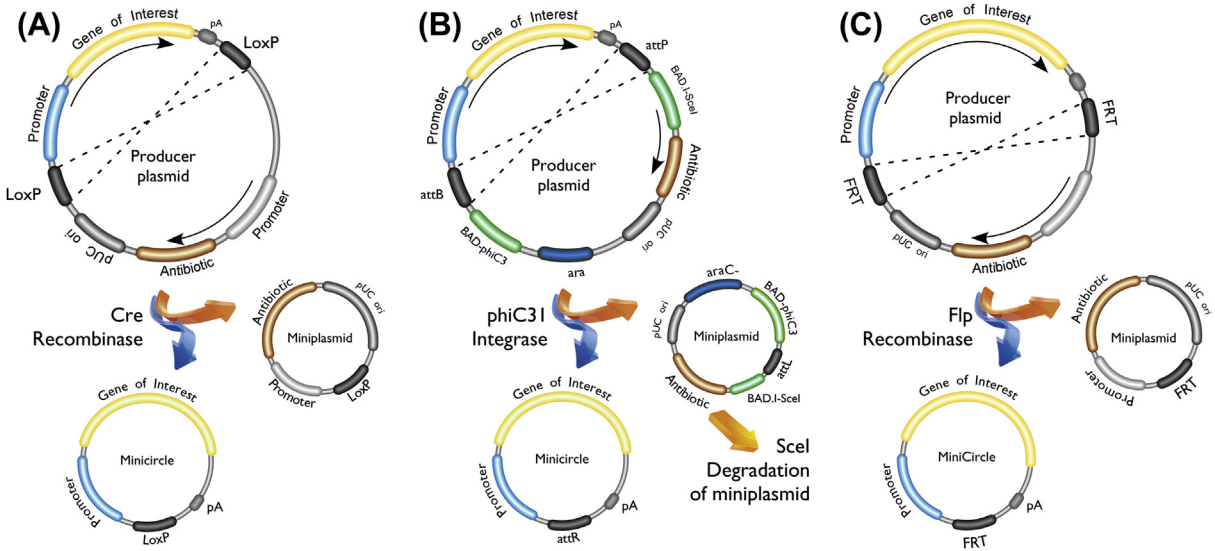

PLATE 11 ***Schematic representation of the three most commonly used methods for minicircle production.*** (A) Generation of minicircles using the Cre/Lox recombination method, where Cre recombinase catalyzes specific recombination of DNA between loxP sites. (B) Generation of minicircles using the C31 integrase, which catalyzes the site-specific recombination of two recognition sites that differ in sequence, typically known as attachment sites attB and attP. Typically, miniplasmids are subsequently digested in vitro by SceI. (C) Generation of minicircles using the Flp recombinase, which catalyzes recombination between the 34-bp-long flippase recognition target (FRT) sites. Table 1 summarizes the application of vectors produced using these systems. (Figure 3 on page 137 of this Volume)

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